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THE UNIVERSITY OF ALBERTA

DEVELOPMENT OF CONTRACTILITY IN THE NEONATAL HEART OF THE RABBIT

By

ZHONG-PING FENG ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

SPRING, 1989

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As the water shapes itself to the vessel that contains it, so a wise man adapts himself to circumstances.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **DEVELOPMENT OF CONTRACTILITY IN THE NEONATAL HEART OF THE RABBIT** submitted by **ZHONG-PING FENG** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE**.

....
(supervisor)

....

....
(Examiners)

Date: April 14, 1989

To my parents

ABSTRACT

The small cardiac reserve in the newborn increases with age to permit a functional modulation of cardiac rate (chronotropism) and contractile force (inotropism) to meet the wide physiological demands after birth. This study tests the hypothesis that a major component of developmental maturation of excitation-contraction (E-C) coupling and control via adrenergic modulation occurs in the postnatal period to accommodate the imposed demands.

Radioligand binding assays were used to determine the numbers of Ca channels ([^{3}H]nitrendipine) and beta-adrenoceptors ([^{3}H]dihydroalprenolol) of ventricular homogenates of rabbit hearts at 3 days, 3 weeks and 3 months after birth. The relative contribution of extracellular and intracellular Ca to activator Ca was studied on steady state and post-rest contractions of ventricular papillary and atrial muscles at the same developmental stages.

Numbers of Ca channels increased 3-fold in the first 3 weeks during which time extracellular Ca contributed a large component of activator Ca. Steady state force was readily increased by increase [Ca]$_o$, stimulation frequency and Ca channel agonist (Bay K8644) in the newborn. With age, inotropic effectiveness of external Ca decreased as the internal Ca stores mature. Intracellular Ca stores also contributed to activator Ca in the immature heart as seen by

the potentiation of post-rest contractions. The internal Ca stores could be increased by raising external Ca, increasing priming frequency and interposing extrasystolic beats which increased this potentiation at a range of different rest intervals. However the capacity of the internal Ca stores was limited in the immature muscle.

Although ryanodine binding sites, the putative intracellular Ca release channels, were present in immature muscles, the contraction of papillary muscles were relatively insensitive to ryanodine blockade suggesting that the channels undergo a postnatal maturation from a neonatal to adult form. Atrial muscles were more mature at birth in the sense that their Ca release sites were already ryanodine-sensitive. Inotropic and chronotropic effects of beta-adrenergic modulation were relatively small at birth in both papillary and atrial muscles, and increased concurrently with incorporation of the receptor number and maturation of the receptor-effector coupling with age. At birth parasympathetic nervous stimulation slowed heart rate but was less effective in its negative inotropic effect of reducing contractile force.

Thus, cardiac reserve of the maturing heart is progressively increased during postnatal development by incorporation of Ca channels, maturation of internal Ca stores and the adrenergic modulation.

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ABBREVIATIONS

[]: concentration;
$[\,]_o$: extracellular concentration
[]i: intracellular concentration
$[^3H]$DHA: $[^3H]$dihydroalprenolol
$[^3H]$NTP: $[^3H]$nitrendipine
ACh: acetylcholine
ATP: adenosine triphosphate
Ca: calcium in any form: free, bound or ionized
Ca^{2+}: ionized calcium.
cAMP: cyclic adenosine 3',5'-monophosphate
DHP: dihydropyridine
E-C coupling: excitation-contraction coupling
ES: extrasystole
F_1: post-rest contractile force
$F_{s.s.}$: steady state contractile force
K: potassium in any form: free, bound or ionized
K^+: ionized potassium
Na: sodium in any form: free, bound or ionized
Na^+: ionized sodium.
PF: priming stimulation frequency
PESP: post-extrasystolic potentiation
SA node: sinoatrial node.
SR: sarcoplasmic reticulum

CHAPTER I. INTRODUCTION

In the mammal, the change from placental to lung gas exchange which occurs at birth, causes profound changes in the circulatory route and the structure of the cardiovascular system (Dawes, 1968). The dynamic change in circulatory pattern from the fetus to the newborn which initiates the transition to adult type circulation is dependent mainly on two events: 1) the removal of the placenta from the systemic circulation, and 2) the aeration of the lungs (Yao, 1983).

PERINATAL CIRCULATION The umbilical venous blood containing relatively high Po_2 leaves the placenta, the site where gas exchange takes place in the mammalian fetus, and enters the inferior vena cava via the ductus venosus. Some of this blood enters the right atrium where it is mixed with the blood from the superior vena cava which is poorly saturated with O_2. Most flows directly into the left atrium through the foramen ovalis and is then distributed to the systemic circulation. Some of the blood in the right heart enters the lung, but most of it bypasses the lung through the ductus arteriosus to supply the rest of the body. The net result of this somewhat complicated arrangement is that the best oxygenated blood reaches the brain and heart, and that the non-gas-exchanging lungs receive only about 15% of the cardiac output (West, 1985).

POSTNATAL CIRCULATION At birth, following the first few breaths, pulmonary vascular resistance falls resulting in an increase in pulmonary blood flow. Following this, left atrial pressure rises and the flap-like foramen ovale quickly closes. The ductus arteriosus constricts a few minutes later partly in response to the direct action of the increased oxygen pressure on its smooth muscles. Flow through the ductus arteriosus soon reverses as the resistance of the pulmonary circulation falls (West, 1985).

Work required of the left and right ventricles is approximately equal as they pump in parallel during fetal life (Rudolph, 1970; Heymann and Rudolph, 1973; Rudolph and Heymann, 1974). With closure of the foramen ovale and ductus arteriosus after birth, blood flow is henceforth in series through the right and left ventricles, resulting in a greater volume work load on each ventricle. In the same period, pulmonary resistance is lowered by expansion of the collapsed lungs (Klopfenstein and Rudolph, 1978; Rudolph, 1979), peripheral resistance increases with loss of the placental circulation (Rudolph, 1970) and the pressure load on the left ventricle becomes significantly greater than that on the right ventricle (Assali et al., 1965). The combination of these effects induces a faster growth in the left ventricular myocardium leading to its relatively large muscle mass as seen in the adult heart.

Thus, development of the myocardium during the early postnatal period accommodates not only the relatively abrupt changes in the patterns of blood flow and circulatory resistance occurring shortly after birth, but also the increasing demand of the rapidly growing animal. In addition to these abrupt and dramatic changes in the route of the circulation during the period of transition to extrauterine life, postnatal development of the myocardium occurs gradually to compensate for the rapidly increased demand of the growing neonate.

1.1. STATEMENT OF PROBLEMS

At the time of birth, the newborn heart contracts with a higher frequency and less regular rate and rhythmicity than the mature heart. In addition, the cardiac output is more dependent on heart rate than stroke volume in the neonate (Gootman, 1983). During postnatal development, the heart rate becomes more consistent and the rhythm more regular. These alterations correspond with an increase in the force of contraction of the heart muscle to maintain a consistent systemic circulation for the increased demand of the growing animal. Therefore, the cardiac cells must mature with age with an increase in the left ventricular muscle mass to ensure a large stroke volume in the adult (Keen 1955; Hort, 1966).

Studies on postnatal development of the mammalian heart have indicated that the immature heart contracts more vigorously and pumps more blood relative to body weight than does the mature heart (Klopfenstein and Rudolph, 1978; Berman and Musselman, 1979; Gilbert, 1980). However, the contractile reserve, i.e. the capability of hearts to increase cardiac output, is considered to increase as animal matures (Klopfenstein et al., 1978; Romero and Friedman, 1979; Gilbert, 1980; Teitel et al., 1985). In addition, the resting cardiac output (Klopfenstein and Rudolph, 1978; Berman and Musselman, 1979; Gilbert, 1980) or the resting contractility (Teitel et al., 1985) is high in the newborn heart and reduced postnatally. Nevertheless, studies on myocardial contractility of isolated cardiac muscles suggested that the maximum contractile force increased with age after birth (cat: Davies et al., 1975. sheep: Friedman, 1972; Anderson et al., 1984. rabbit: Jarmakani et al., 1982; Nakanishi and Jarmakani, 1984). The contractile velocity also increased though duration of the contraction remained unchanged during postnatal development (Friedman, 1972; Davies et al., 1975; Jarmakani et al., 1982; Nakanishi and Jarmakani, 1984). Moreover, the neonatal heart is less sensitive than the adult heart to numerous antiarrhythmic (Gelband et al., 1971; Goldberg et al., 1975; Mary-Rabine and Rosen, 1978; Ezrin et al., 1980), and inotropic drugs (Brus and Jacobowitz, 1971; Gillette et al., 1976; Toda, 1980; Urthaler et al., 1980). Explanations for these age-related alterations include that 1) immaturity of the autonomic nervous system and autonomic receptors (Geis et al., 1975; Pappano, 1977; Vlk and Vincenzi, 1977); 2) pharmacokinetic factors affecting drug uptake and distribution

(Gelband et al., 1971; Udkow, 1978); 3) ultrastructural properties related to drug and ionic permeation (Langer et al., 1975; Legato, 1979a); and 4) the influence of immature cellular metabolism on transmembrane permeability and ionic gradients (Bernard, 1976; Rosen et al., 1975, 1977; Mary-Rabine and Rosen, 1978; Toda, 1980).

However, relatively less is known about the age-related contractile reserve and its underlying mechanisms. One possible explanation is that Ca storage capacity in the SR is very low and the reliance of the muscle contraction on Ca released from the SR is small at the time of birth. Thus the myocardial contraction of the newborn must depend on external Ca which enters the cell via the sarcolemmal Ca channels. The small myocardial contractile reserve in the newborn, therefore, could be due to a transition from external to internal Ca sources and a more limited recirculation of the intracellular Ca. In addition, postnatal development of the sympathetic modulation which allows further increase in the contractile reserve by increasing amount of activate Ca may also occur concurrently. To test these possibilities, we have in this study characterized myocardial contractile behaviour in isolated atrial and ventricular papillary muscles from rabbits during postnatal development to seek the underlying mechanisms.

1.2. REVIEW OF THE LITERATURE (BACKGROUND)

The heart is a hollow muscular pump which rhythmically propels blood around the circulation to deliver nutrients and remove wastes from each of the organs, to transport hormones and other regulatory substances between various regions of the body. In order to control and modulate its own pumping action to meet the constantly changing demands of the body, the heart possesses three fundamental properties to initiate excitatory impulses at regular intervals (automaticity), to propagate the impulses to other cells (conductivity) and to shorten the myocytes in response to a stimulus (contractility). The autonomic innervation of heart plays a modulatory role to increase or decrease these cardiac functions.

It has long been recognized that calcium (Ca) is fundamental to the contractile process in cardiac muscle since the classical studies of Ringer (1883). Nevertheless, the exact mechanisms of excitation-contraction coupling (E-C coupling) are not yet fully understood. In adult cardiac cells, extracellular Ca enters the cells during the action potential via the voltage-dependent Ca channels in the sarcolemma. The inward Ca current triggers a release of activated Ca from the SR (and/or other intracellular Ca stores) (Nayler and Merrilees, 1971; Fabiato and Fabiato, 1977, 1979; Fabiato, 1983). The activated Ca binds to the Ca binding sites on troponin C to remove an inhibitory effect of tropomyosin on the association of myosin cross bridges with at actin (Winegrad, 1984). Attachment of actin to myosin follows. Cross bridge cycling occurs as a result of ATP breakdown by myosin ATPase and phosphate release, and muscle contracts. The force of contraction is proportional to the number of cross-bridges made during this process and this in turn is proportional to the concentration of free Ca ions in the interior of the cell (reviewed by McCall, 1987). Relaxation of the muscle occurs as Ca ions dissociate from troponin. Subsequently the Ca is sequestered by the SR via ATP-dependent Ca pump on the SR (reviewed by Chapman, 1979; Fabiato and Fabiato, 1979) and extruded from the cell via Na/Ca exchange (Horackova and Vassort, 1979) and ATP-dependent Ca pump on the sarcolemma (Ziegelhoffer et al., 1979).

The postnatal development of the mammalian heart has been studied extensively using various species at different age. This review will focus mainly on the myocardial contractility and its regulatory mechanisms which include the basic contractile properties inherent in the muscle, excitation-contraction coupling mediated by Ca cycling and modulations of autonomic nervous system.

1.2.1. Postnatal morphological changes in immature myocytes

With age, the subcellular organelles of ventricular cells appear to mature to meet the requirements of increasing heart function and demand of body. In atria, the developmental changes in ultrastructure is similar to that of ventricle, but the atrial cells grow more slowly than ventricular cells. The differences between atrium and ventricle become more obvious with age (Hirakow and Gotoh, 1980; Gotoh, 1983).

1.2.1.1. Volume and number of myocytes

Myocyte volume increases during postnatal development in rat and rabbit, with an increase in cell length. Since the increase in cell volume is greater than the increase in cross-sectional area, the surface-to-volume ratio appears to decrease (Stewart and Page, 1978; Anversa et al., 1980). Cell length-to-width ratio, which is variable in immature myocytes in fetal hearts, is constant in the neonatal heart from birth to 30 day-old (Korecky and Rakusan, 1978).

Cell number also increases during early postnatal development (rat within 17-18 day-old; dog within 4-6 week-old) in left ventricle but not in right ventricle (Bishop and Hine, 1975; Rumyantsev, 1978). It was suggested that both hyperplasia and hypertrophy occur in the left ventricle in response to increased pressure load (afterload) during postnatal life, while only hypertrophy occurs in the right ventricle due to the higher volume load (preload) (Anversa et al., 1980). Arrangement of myofilaments around the Z line is quite irregular as compared with the gradual organized arrangement of myofilaments in the 3 week-old neonate (Hopkins et al., 1973; Legato 1975, 1979a, 1979b; Anversa et al., 1981; Carlsson et al., 1982).

The cardiac muscle fiber is enveloped by a cell membrane called the sarcolemma which has T-tubules opening to the extracellular space. The intracellular membrane system, the sarcoplasmic reticulum (SR) is closely opposed to the sarcolemma and T tubules. The sarcolemma is the boundary between high, millimolar Ca concentrations in the extracellular space (1.5 to 2 mM) and extremely low Ca concentrations (0.1 uM) in the cytosol of the resting myocardium (McCall, 1987). The sarcolemma regulates cellular Ca concentration through mechanisms that involve voltage-dependent Ca channels (Reuter, 1979), the Na/Ca exchange system (Reeves, 1985) and an ATP-dependent Ca pump (Carafoli and Zurini, 1982). The SR functions as intracellular Ca stores and controls the intracellular Ca recycling through a ryanodine sensitive Ca channel (Fill and Coronado, 1988) to release Ca which initiates muscle contraction and an ATP-dependent Ca pump to take up Ca during muscle relaxation (Martonosi et al., 1978; Fabiato, 1982).

1.2.1.2.T-tubular system

In most animals, there are no T-tubules present at birth (rat: Carlsson et al., 1982. rabbit: Hoerter et al., 1981. dog: Legato, 1979b. cat: Sheridan et al., 1977; Gotoh 1983. hamster: Colgan et al., 1978. exception is guinea pig: Forbes and Sperelakis, 1976; Hirakow and Gotoh, 1980) until 3 weeks to 5 months (dependent on the species). The emerging T-tubules penetrate and divide the cell into "units" of similar size during development (Page et al., 1974).

1.2.1.3. The sarcoplasmic reticulum (SR)

The SR which has the function to store, activate and transport Ca, is seen at birth in all mammalian species. Increases in volume and membrane surface area of the SR occur concurrently with an increase in myofibril volume (Page et al., 1974; Colgan et al., 1978; Olivetti et al., 1980; Hoerter et al., 1981).

1.2.1.4. Mitochondria

Mitochondria are responsible for production of the high energy phosphate (ATP) in cardiac cells. The mitochondrial Ca transport system becomes important only in pathophysiological conditions where there is an abnormal influx of Ca into the cell, such as in ischemia reperfusion (McCormack et al., 1988). Mitochondria are irregular in shape and size with few cristae (Legato, 1975 and 1979b) in the newborn, become more uniform but complex size and shape, and contain progressively more packed and longer cristae during postnatal development (Smith and Page, 1977; Legato, 1979b).

1.2.2. Myofibrillar proteins in immature hearts

The myofibrils contain thick filaments, which are composed largely of myosin and contribute both its darkly staining characteristics and high birefringence, and thin filaments which are composed of actin and troponin-tropomyosin complex and contribute both their light staining and regulatory property (Katz, 1977). The myofilaments are smaller in diameter and less amounts in neonatal cardiac cells than that in adult cat (Maylie, 1982). In addition, the content of myofilaments and the activity of myofibrillar ATPase increases after birth and reach a peak in the adult heart (Rabbit: Naylar and Fassold, 1977; Nakanishi and Jarmakani, 1984. Cat: Maylie, 1982).

1.2.2.1. Myosin

Myosin is a large (molecular weight 480,000 dalton), elongated molecule with a globular head and a long alpha helical tail (Martin et al., 1981). Light meromyosin (LMM) comprises most of the long chain coil and heavy meromyosin (HMM), a short length of the chain and the globular head (Penenfsky 1983). The HMM subunit is an ATP dependent enzyme and has the ability to hydrolyze the polyphosphate chain of ATP. Myosin ATPase activity correlates directly to the maximal muscle shortening velocity (Katz, 1977). The myosin tails are wound together to form a rigid backbone, from which myosin heads project to make up the cross-bridges to react with actin.

Myosin ATPase activity is low in the newborn heart and increases with age in pig and rat hearts (Syrovy, 1982). In addition, the isoenzymes of myosin have been reported to shift from a predominantly slow type (V3) to a fast V1 type (the most active ATPase) during postnatal development. The increase in shortening velocity during development from neonatal to adult heart, therefore, probably results from the increased amount of the fast V1 type myosin isoenzyme and a disappearance of V3 (the slowest ATPase) following a conversion form (V2) in the adult (Sheep: Friedman, 1972. Rat: Hopkins et al., 1973. Rabbit: Jarmakani et al., 1982; Nakanishi and Jarmakani, 1984).

1.2.2.2. Actin

Actin (molecular weight 42,000 dalton) is formed by a ATP-dependent aggregation of globular actin monomers. It interacts physiochemically with tropomyosin and directly activates myosin ATPase activity (Katz, 1970).

1.2.2.3. Tropomyosin

Tropomyosin contains two peptide chains linked by a single disulfied bond in a coiled coil conformation. Combined with actin, tropomyosin plays a very important role in modulating the interactions between actin and myosin during contraction (Ebashi, 1974; Katz, 1977).

1.2.2.4. Troponin

Troponin composes of three subunits: 1) troponin I (TnI), which has the ability to inhibit the interactions between actin and myosin (Kranias and Solaso, 1982); 2) troponin T (TnT), which serves primarily to bind the troponin complex to tropomyosin (Johnson et al., 1981), and 3) troponin C (TnC), which contains two high (0.03 uM) and two or one low-affinity (3 uM) binding sites for calcium and binds three molecules of calcium per molecule of troponin complex by its two different type calcium binding sites (Holroyde et al., 1980; Johnson et al., 1980). The low-affinity site is Ca-specific and have a regulatory function (Potter et al.,1981; Robertson et al., 1981). The ability of troponin C to bind calcium is regulated by the phosphorylation of TnI via a cyclic AMP-dependent process (Ray et al., 1976; Perry, 1979; Kranias 1982). The action of TnI-TnC complex results in a decline of their ability to bind Ca released during excitation-contraction coupling and thus to activate the contractile events of the heart. TnT is an unclear subunit.

TnC isolated from fetal rat heart has the same binding properties as that from the adult, but the calcium sensitivity of TnC decreases in the newborn heart (Rockstein, 1978). It is not clear whether the decline in Ca sensitivity in rat hearts is due to developmental changes in the properties of troponin C. In addition, the hearts of the fetal and newborn (<9 day-old) rat do not contain the adult form of TnI. Two unidentified proteins migrate in the region of the adult TnI.

1.2.3. Excitation-contraction coupling in heart cells

Ca movement in myocardial cells during excitation-contraction (E-C) coupling include 1) Ca influx across the sarcolemma by slow inward current via a voltage dependent Ca channel; 2) intracellular Ca recycling by which Ca release and uptake by the sarcoplasmic reticulum (SR); and 3) Ca efflux across the sarcolemma by Na/Ca exchanger and ATP-dependent Ca pump.

1.2.3.1. Voltage-dependent Ca channels on the sarcolemma

Ca channels in vertebrates have been identified as three classes, T (transient), N (neuron) and L (large) recently (Tsien, 1983). In the heart, most channels are the L type which is sensitive to 1,4-dihydropyridines. The density of the L type channel in cardiac cell membranes has been estimated as 1,000-10,1000 per cell, or 0.5 - 5 channels/um^2 (Bean, 1983; Reuter, 1983).

Different dihydropyridines have unique effect on channel gating (Hille, 1984; Schwartz and Triggle, 1984; Hamilton et al., 1987), although they act primarily on a single site in L-type voltage-dependent Ca channels (Nowychy et al., 1985). According to the model behavior of the L-type channel (Hess et al., 1984), nifedipine and nitrendipine have a high affinity to mode 0 (70% of channels are inactivated) to close the channels, therefore to reduce the inward Ca current in heart muscles (Lee and Tsien, 1983; Bean, 1984). In contrast, Bay K8644 acts on mode 2 (long openings punctuated by the briefest closures) to increase the chance of the channel operating and consequently increases the trans-sarcolemmal Ca influx (Kokubun and Reuter, 1984; Sangninetti and Kass, 1984; Thomas et al., 1987).

Modulation of Ca channel gating involves a cAMP dependent phosphorylation of channel proteins (Rinaldi et al., 1982; Hagiwara, 1983; Reuter, 1983). Although the nature of the postnatal change in the calcium channel is unknown, one possibility includes the maturation of second messenger systems. There is good evidence that the basal myocardial adenylate cyclase activity increases progressively with postnatal age (Vulliemoz et al., 1984), which may contribute to developmental changes in the gating properties of calcium channels.

In the adult cells, the Ca channels are concentrated in the T-tubules of the sarcolemma. It has been reported that the voltage-dependent Ca channel appears to be present in the neonatal myocytes (Penefsky et al., 1980; Van Ginneken and Jongsma, 1983). Since the T system is absent in early development of mammalian hearts, the channel may be dispersed along the sarcolemma.

1.2.3.2. Ca influx via the sarcolemmal ca channel

Entry of Ca from the extracellular space to the cytosol, facilitated by the specific voltage dependent Ca channel (Katz, 1983), occurs during plateau phase of the action potential. The Ca inward current can be blocked by several ions (Mn^{++}, La^{+++}) and drugs (i.e. nifedipine, verapamil), and is enhanced by cetacholamines (Reuter and Scholz, 1977; Zsoter, 1980; Mitchell, et al., 1983). The Ca influx also depends on the extracellular Ca concentration ($[Ca]_o$) and the voltage or receptor mediated opening of Ca channels (Vaughan-Jones, 1986).

Jarmakani et al. (1982) reported that the maximal increase in positive inotropic response of adult heart muscles to $[Ca]_o$ was greater and occurred at less concentration of $[Ca]_o$ than that in the neonate. In contrast, Boucek et al. (1984) and Artman et al. (1985) have shown that the immature rabbit heart was more sensitivity to Ca channel blockers (verapamil, nifedipine and diltiazem) in comparison to the adult, showing a greater inhibition of contractility. Nevertheless, total Ca influx at any $[Ca]_o$ was the same in the neonate and adult rabbit heart (Jarmakani et al., 1982). These conflicting results may suggest that 1) number of Ca channels may increase with age because of the greater effectiveness of Ca channel blockers; 2) effectiveness of Ca once which penetrates the membrane in activating muscle increases with age possibly due to ability to release intracellular calcium from the stores. These intracellular Ca stores are known to be small initially at birth and to develop during postnatal development (see next section), and 3) Ca channel gating in neonate heart may have a higher capacity to allow more Ca ions entry per channel.

1.2.3.3. Intracellular calcium recycling in the SR

Ultrastructural studies have revealed that the volume and membrane area of the longitudinal tubules of the SR increase during the early postnatal development of the rat (Page et al., 1974; Rumyantsev, 1978; Olivetti et al., 1980), hamster (Colgan et al., 1978), rabbit (Hoerter et al., 1981), cat (Maylie, 1982) and dog (Bishop et al., 1975). The T tubule network of the myocardium was absent at birth in the rat (Carlsson et al., 1982), hamster (Colgan et al., 1978), rabbit (Hoerter et al., 1981), cat (Sheridan et al., 1977; Maylie, 1982; Gotoh, 1983) and dog (Legato, 1979b) except guinea-pig (Forbes and Sperelakis, 1976; Hirakow and Gotoh, 1980). Generally, the T system appeared and matured at 3 weeks to 5 months after birth depending on the species.

The ability of the SR to release and take up Ca is poorly developed in the newborn heart (Rabbit: Seguchi et al., 1986. Cat: Nayler and Fassold, 1977; Maylie, 1982. Rat: Fabiato and Fabiato, 1978). Postnatal changes in cardiac contractile responses to extracellular Ca (Nishioka et al., 1981; Park et al., 1980), lanthanum (George et al., 1983), manganese (George et al., 1983) and caffeine (George et al., 1984) suggest that two sources of activator Ca (extracellular and intracellular sources) may contribute to the development of tension in neonatal and adult heart. Compared to the adult, transmembrane

Ca influx in the neonate may be sufficient to activate myofibrils in small diameter cells and appears to be the major source of Ca for the activation prior to development of the SR and T-tubule network. In contrast, the activator Ca in adult heart is generally believed to be release from the SR, which is triggered by trans-sarcolemmal Ca influx.

1.2.3.4. Na/Ca exchanger

The sarcolemmal Na/Ca exchanger is likely to play a key role in dictating Ca flux across this membrane, and consequently the intracellular concentration of Ca and hence contractile function (McCormack et al., 1988). The exchanger may operate to promote Ca efflux or Ca influx, depending on the trans-sarcolemmal electrochemical gradients for Na and Ca. Studies on Na/Ca exchanger have been limited since the noticeable lack of any convincing pharmacology for this exchanger protein, and the difficulty to purify this protein or other biochemical properties.

1.2.3.5. Na/K pump

The sarcolemmal Na/K ATPase functions to pump K into the cell and Na out to maintain the necessary concentration gradients of the ions (Schwartz et al., 1972; Glitsch, 1979). Since the concentration gradient of Na is important for Ca extrusion via Na/Ca exchanger, accumulation of intracellular Na by cardiac glycosides which block selectively the Na/K

ATPase, leads directly intracellular Ca accumulation, and therefore increases cardiac contractility (positive inotropic effect) (Eisner et al., 1984; Lee, 1985). The activity and density of the Na/K ATPase are high in the fetal and neonatal hearts and decrease progressively as animal matures (Sperelakis, 1972; Langer et al., 1975; Marsh et al., 1981).

1.2.4. Autonomic modulation of cardiac function

Cardiac function is regulated by autonomic innervation. Stimulation of the sympathetic nerve terminals leads to release of cetacholamines which act on adrenoceptors and cause an increase in heart rate (positive chronotropic effect) and an increase in the force of contraction (positive inotropic effect). Conversely, activation of parasympathetic nerves releases acetylcholine (ACh) which binds on cholinergic muscarinic-receptor and induces negative chronotropic and inotropic effects (James, 1967).

1.2.4.1. Adrenergic receptors

The adrenergic receptors were termed alpha and beta subtypes in 1948 (Ahlquist). Alpha receptors are subclassified into alpha-1 and alpha-2 types (Berthelson and Pettinger, 1977) and beta receptors into beta-1 and beta-2 types (Lands et al., 1967). These later subtypes of adrenoceptors are identified in heart and mediate positive inotropic and chronotropic modulations of cardiac functions (Scholz, 1980).

BETA-ADRENOCEPTORS Both beta-1 and beta-2 receptors are present in the ventricles and atria (Brodde, 1986). Beta-1 receptor is more effective in regulation of contractile force, and beta-2 regulates both contraction and rhythm of heart. The activation of beta-adrenoceptors in heart is accompanied by an increase in the adenylate cyclase activity to elevate cyclic adenosine 3',5'-monophosphate (cAMP) levels in the cell, by activating the guanine nucleotide regulatory protein (Pappono, 1981). This results in an increase in Ca influx by phosphorylating sarcolemmal Ca channel proteins, abbreviates the time to peak tension, and increases the rate of Ca uptake by the SR.

Adrenaline accelerated the heart beat at the second embryonic day of chick (Markowitz, 1931) by interacting with the beta-adrenoceptor (McCarty, 1960). Binding studies suggests that beta-receptors are present at fifth day in embryonic chick (Alexander et al., 1982) and at ninth day in the embryonic mouse heart cells (Lane et al., 1977). However, a significant receptor site binding to [^{3}H]dihydroalprenolol ([^{3}H]DHA) with no positive chronotropic effect by isoproterenol was reported by Chen et al. (1979).

The beta-adrenoceptor in the mouse heart showed an increase between 17 days in utero and 3 days after birth, no change between 3-14 days after birth, and a eventual decrease to adult level (Roeske and Wildenthal, 1981). As in the rat and chick, the decrease in beta-receptors that occurred between 2 weeks after birth and adulthood was attributed to "down" regulation by virtue of secretion of noradrenaline from newly developed sympathetic adrenergic nerve (Roeske and Wildenthal, 1981).

ALPHA-ADRENOCEPTORS Alpha-1 receptors, located on the postsynaptic region, are present in vascular smooth muscles in the heart in a large proportion. Alpha-1 receptors are also present in the mammalian myocardium (Benfey, 1982) although their effect has not been fully understood (Giotti et al., 1983). The positive inotropic effect of alpha-1 receptor were observed in ventricles (Amerini et al., 1985) and atria (Hashimoto et al., 1983) of rats, and this effect was shown to be very potent in the young animal but decreases with age. Alpha-2 receptors are found on the presynaptic region on the nerve and modulate the release of the neurotransmitters via a negative feedback mechanism (Wikberg, 1978).

1.2.4.2. Muscarinic cholinergic receptors

Interaction between ACh and its muscarinic receptor on the heart reduces the intracellular cAMP levels by activating Gi protein to generate the negative chronotropic and inotropic effect (Hulme et al., 1981; Linden et al., 1982). Since the early work of Hsu (1933) and Cullis and Lucas (1936) who showed that the responses of the embryonic heart cells to ACh varied with age, muscarinic receptors and underlying transduction mechanism have been suggested to be age-related.

In the young ventricular cells of chick, ACh has a little of no effect on shortening the action potential, whereas the atrial action potential is markedly shortened (Pappano, 1972; Loffelholz et al., 1974; Sperelakis and Pappano, 1983). The specific binding studies of [^{3}H]QNB (quinuclinyl benzilate, a highly specific muscarinic antagonist) was obtained in 3 day chick embryo hearts (Galper, 1977) and in 9 day embryo mouse hearts (Lane, 1977). However, the concentration of the [^{3}H]QNB binding sites in rat atrial muscles decreased progressively during postnatal development (Nedoma et al., 1986).

1.3. HYPOTHESIS

Myocardial contractile behavior undergoes a postnatal development as animal matures. The age-dependent alterations would be directly related to excitation-contraction coupling system and autonomic modulatory mechanisms.

1.4. AIMS OF THE THESIS

The aims of this study were to:

1) Characterize and compare myocardial contractile function in postnatal developing mammalian hearts;

2) Delineate underlying mechanisms for the age-related differences in contractile behaviour in ventricular and atrial muscles, which include:

   a) relative contribution of external Ca to force generation to the immature heart;

b) relative contribution of internal Ca to force generation in the immature heart;

3) Delineate the regulation of myocardial contraction by beta-adrenoceptor and cholinergic muscarinic receptor stimulations.

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CHAPTER II. GENERAL METHODS

2.1. ANIMAL MODEL

Rabbits were used in our studies of the development cardiac contractility.

The Dutch rabbits were considered as the optimal model since 1) myocardial contractility can be studied easily at many stages of development in contrast to the guinea pig, which has short development cycle with a unclear age stages; 2) they are inexpensive compared to the dog, although which also has a very long developmental cycle; 3) their cardiac function is more closely related to human than the rat. The latter's heart generates large contractile tension at low frequency of stimulation and the tension is reduced as increase in the rate of stimulation (Hoffmann and Kelly, 1959; Forester and Mainwood, 1974).

Dutch rabbits were divided to three age groups. The newborn group was selected as 1-3 days after birth since there is little or no participation of the SR in contraction at this time (Fabiato and Fabiato, 1978, Maylie, 1982). The immature group was aged 20-22 days at which time the growing rabbits are weaned from the mother. Some evidence suggested there is limited participation of the SR in contraction at first 3 weeks of life (Boucek et al., 1984; George and Jarmakani, 1983). The young adult was aged 2-4 months old by which time the heart was considerated functionally mature.

2.2. RADIOLIGAND BINDING TO MUSCLE HOMOGENATES IN QUANTIFICATION OF RECEPTOR AND ION CHANNEL NUMBERS*

Bambrick LL, Howlett SE, Feng Z-P and Gordon T

2.2.1. Introduction

The number of receptor and ion channel proteins, which is of particular interest in the study of muscle plasticity in disease, development and experimental manipulation, is readily quantified using radioligand binding assays (Bambrick and Gordon, 1987; Howlett and Gordon, 1987; Wagner et al., 1986; Rogart and Regan, 1985; Erman et al., 1983; Sherman and Catterall, 1982; Catterall and Coppersmith, 1981; Renaud et al., 1981; Chen et al., 1979). The simplest approach is to conduct these assays with muscle homogenate preparations which retain all the membrane binding sites. The high and reproducible recovery of sites, which is in contrast to the variable recovery in more purified membrane preparations, allows quantification of the total number of binding sites in whole muscle. Numbers of radioligand binding sites can then be normalized by muscle weight, protein content, and/or fibre diameter for comparison of muscles in different treatment groups. Binding studies using homogenate preparations have a number of other advantages which include: ease of muscle preparation, reproducibility, minimal tissue requirement and good retention of native receptor properties during homogenate preparation. There are methodological problems, however. The retention of all cellular comonents is conductive to non-specific ligand binding which severely limits the resolution of specific binding sites (Manalan et al., 1984). In addition, tissue heterogeneity may be a further complicating factor (Catterall and Coppersmith, 1981; Manalan et al., 1984). If these problems are solved, binding assays with homogenate preparations become a powerful means of studying the plasticity and control of receptors and ion channels in muscle.

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* A condensed version of this section has appeared in a paper published in J. Pharmacol. Method. [Bambrick LL, Howlett SE, Feng Z-P and Gordon T. Radioligand binding to muscle homogenates to quantify receptor and ion channel numbers. J. Pharmacol. Method. 1989, 20:313-321.].

We address these problems with the aim of providing a useful framework for adapting homogenate binding for a number of different radioligands such the membrane proteins can be quantitatively and reproducibly assayed under a wide variety of experimental conditions. The optimization of radioligand binding assays for several ion channels and receptors in investigations of normal, developing, diseased and experimentally-manipulated muscles is described.

2.2.2. Methods

2.2.2.1. Preparation of muscle homogenates

Dutch rabbits (1.5-2.0 Kg, Biological Animal Services, University of Alberta) were killed by cervical dislocation. Hearts were rapidly removed, weighed and placed in ice-cold 250 mM sucrose and 20 mM HEPES buffer (pH 7.8). The muscle of interest was minced and then homogenized with a Brinkmann Polytron at half the maximum speed for two 10 sec bursts. The homogenate was filtered through 4 layers of cheese cloth to remove large connective tissue fragments and used either immediately or after storage at -70°C. Preparation in this fashion minimizes alterations in native receptor properties (Manalan et al., 1984).

2.2.2.2. Radioligand binding assays

[^{3}H]dihydroalprenolol (specific activity of 42 Ci/mmol, radiochemical purity greater than 98.5%) and ^{3}H-nitrendipine (77.4-85.0 Ci/mmol, 99%, stored and used in light-protected conditions to prevent degradation) were obtained from New England Nuclear.

Assays were conducted using standard techniques and appropriate conditions for each ligand (as described in detail by Bennett and Yamamura, 1985). These conditions have to be modified for high resolution of specific binding sites to muscle homogenates. Methods for establishing these conditions are described below and form the basis for the results and discussion section. Binding was initiated by adding muscle protein to test tubes containing buffer of the following composition and total volume: 50 mM Tris-HCl (pH 7.4) for assays with [^{3}H]NTP (2 ml) and [^{3}H]DHA (0.2 ml). Total and nonspecific binding were determined in the absence or presence respectively, of unlabelled ligand in concentrations 1000-fold the K_d of labelled ligand. Each concentration was determined directly by taking small aliquots of the incubation medium for radioactive counting. Duplicate assays were performed for a range of labelled ligand concentrations from 10 times less than to 5 - 10 times more than the K_d of the ligand. The reaction was terminated by rapid vacuum filtration through filters which were presoaked in buffer. The filters, which retained the muscle homogenate, were then washed with cold buffer 0.8 times. Optimum concentrations of protein and of unlabelled competitive ligand, incubation time, choice of filter, composition of buffer for filter soaking and number of filter washes were established, as described in section 2.2.3.3., prior to the equilibrium binding assays. The filters and aliquots were transferred to glass or plastic scintillation vials containing Aquasol (New England Nuclear) for tritium counting. Radioactive counts per minute (cpm) were measured, at an efficiency of about 45%, using a Beckmann LS 7500 scintillation counter. The free radioligand concentration was obtained by subtracting the total homogenate bound from the total radioligand concentration in the incubation medium as determined from the aliquots.

(Figure 2.1. near here)

Total and non-specific binding were plotted as a function of the free radioligand concentration as shown in the example in Figure 2.1A. Specific binding was obtained by subtracting the linear, non-specific component from the total binding and fitted, as shown in Figure 2.1B, using least mean squares criteria (Hartley, 1961) with a hyperbolic curve of the form:

$$B = \frac{B_{max} \cdot [F]}{[F] + K_d}$$

where B is the amount of bound ligand, F is the concentration of free ligand, B_{max} is the maximum number of binding sites and K_d is the dissociation constant. B_{max} and K_d were also calculated by replotting the data as Scatchard plots (Figure 2.1C), where the slope is $-1/K_d$ and the x intercept is the B_{max}.

2.2.3.3. Establishment of radioligand binding assay protocol

A single concentration of labelled ligand 3-5 times higher than the previously reported values of the K_d was used to establish the optimal conditions for equilibrium binding assays using muscle homogenates.

MUSCLE HOMOGENATE PROTEIN CONCENTRATION

Relatively large amounts of protein are required for binding assays since the receptor content of crude homogenates is relatively low. The optimum protein concentration measured using the method of Lowry et al., (1951) with bovine serum albumin (BSA) as the standard, was established to meet 3 criteria: 1) high resolution of specific binding, 2) optimum binding site concentration such that the free radioligand concentration is far in excess of the amount bound as required for equilibrium binding, and 3) rapid vacuum filtration. For example, 20 nM [^{125}I]BTX was incubated with increasing concentrations of skeletal muscle homogenate diluted in 50 mM sodium phosphate buffer (pH 7.4) in the presence or absence of 4 uM BTX to determine the total and non-specific binding, respectively, as shown in Figure 2.2A. All binding was linearly related to protein concentration and specific binding was obtained by subtraction of the non-specific from the total binding. Note that in this example there is considerable non-specific binding of the radioligand to the filters in the absence of protein. This is discussed in detail in the results and discussion section. All subsequent binding assays were carried out at the optimum concentration of muscle protein within this linear range; in the example shown (Figure 2.2A) this was from 0.6 - 0.9 mg homogenate protein.

(Figure 2.2. near here)

DISPLACEMENT OF RADIOLIGAND WITH UNLABELLED (COMPETITIVE) LIGAND

To ensure that sufficient unlabelled ligand was used to displace all the specific binding, total binding of a given concentration of radioligand was measured in the presence of increasing concentrations of a competitive unlabelled ligand.This is shown for nifedipine displacement of [^{3}H]NTP binding to adult cardiac muscle in Figure 2.2B. As illustrated in Figure 2.2B, the binding remaining is the non-specific binding at this concentration of radioligand.

Typically, specific binding to a single site is progressively displaced over about a 100-fold concentration range of unlabelled ligand. By transforming these data using the legit transform method of Rodbard and Frazier (1975) the concentration of unlabelled drug producing 50% inhibition of specific binding (the IC_{50} value) was calculated. Thus, the affinity constant for the unlabelled drug or K_i, was determined from the following equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1+[L]/K_d}$$

where [L] is the concentration of labelled ligand used in the assay. This analysis of K_i permits the assessment of rank orders of potency of drugs.

ASSOCIATION AND DISSOCIATION OF RADIOLIGAND TO BINDING SITES

The rates of association and dissociation, which differ according to the ligand, were examined to establish the required incubation and filtration times. Since the K_d is the ratio of the rate of dissociation (K_{-1}) to the rate of association (K_{+1}) (for R+L=RL, where R is the Ligand binding site), the rate constants were used to provide another measure of ligand affinity. Optimal concentrations of muscle protein and radioligand were chosen to ensure that less than 10% of the initial free ligand was bound at equilibrium so that conditions for pseudo-first order kinetics were satisfied.

To obtain the net rate of formation of the ligand-binding site complex (K_{obs}), one concentration of radioligand was incubated with an aliquot of muscle homogenate in the presence or absence of unlabelled competing ligand, and the reaction was terminated at progressively longer times. The dissociation rate (K_{-1}) was determined by incubating aliquots of muscle protein with radioligand for sufficient time to establish equilibrium after which a 1,000-fold excess of competing ligand was added to the incubate. Figure 2.2C shows the association and dissociation curves for [^{3}H]NTP. The values of K_{obs} and K_{-1} were measured using a logarithmic transform method (Bennett and Yamamura, 1985), and the value of K_{+1} was computed from the following equation:

$$K_1 = \frac{K_{obs} - K_{-1}}{[L]}$$

where [L] is the concentration of ligand used in the assay. By allowing long incubation times, the possibility of receptor degradation by proteolysis was also assessed. A progressive decrease in the plateau with time could indicate receptor degradation.

FILTERS AND FILTER WASHING

The filters of choice in many radioligand binding assays are glass fibre filters with different loading capacities (eg. Whatman GF/B and GF/C) and these were chosen initially for equilibrium binding assays. To decrease the non-specific binding, a free concentration of radioligand which produced 70-80% of the B_{max} was used to test the effect of different filters on total, non-specific and specific binding in the presence or absence of muscle homogenate. Filters, filter wash times and buffer solutions were systematically investigated (eg. Figure 2.2D and 2.2.3. Results and discussion).

2.2.3. Results and discussion

2.2.3.1. Resolution of binding in equilibrium binding assays

NON-SPECIFIC BINDING

There are at least three quite different sources of non-specific binding in filtration assays. These include 1) ligand adsorption, particularly in the case of lipophilic ligands such as [^{3}H]NTP, by muscle binding sites which do not correspond to the specific receptors or channels of interest, 2) trapping of the ligand by the homogenate during filtration, and ligand binding to the filters used to collect the homogenate after incubation.

One major problem of using muscle homogenates is the low concentration of specific binding sites and the concomitant relative increase in non-specific sites. The problems incurred with high non-specific binding are shown in Figure 2.3A in an example of equilibrium binding of [^{3}H]NTP to calcium channels in a homogenate preparation of adult rabbit hearts. The total binding shown by the + symbol includes a saturable, specific component (a hyperbolic function) and a non-specific component (a linear function). The latter is the major component of the total binding shown in this example and markedly reduces the ability to resolve the specific binding shown by the X symbols in the figure. The uncertainty of the measurement is well-illustrated by the poor fit of the data in the Scatchard plot shown in Figure 2.3B (cf. Figure 2.7A and B). Thus, a major limitation in binding to homogenate preparations is the high non-specific ligand binding.

(Figure 2.3. near here)

The non-specific binding can be reduced, without affecting the specific binding, by repeated washing of the filters with buffer solution. This is only true when the dissociation rate is long with respect to the wash time. When the rate of dissociation is rapid, it is important to separate the protein rapidly following termination of binding. Thus, washing the filters to reduce non-specific binding may result in the loss of radioligand from specific binding sites during the wash time. As shown in Figure 2.4., the resolution of the specific [^{3}H]NTP binding from the total can be dramatically improved by increasing the number of times the filters are washed. In this example using guinea pig ileum, there is little further improvement beyond 5 - 6 washes. Yet, for another tritiated ligand, [^{3}H]DHA, more than single wash was ineffective in reducing the non-specific binding.

(Figure 2.4. near here)

SPURIOUS SPECIFIC BINDING

While non-specific filter binding decreases the resolution of specific ligand binding to biologic sites, specific binding to filters actually generates artificial data as illustrated in Figure 2.5A and B which show propranolol displaced, specific binding of [^{3}H]DHA to several types of filters in the absence of muscle homogenates. This spurious binding of [^{3}H]DHA was sufficiently high to interfere with the resolution of specific binding of [^{3}H]DHA to beta-adrenoceptors. This spurious specific binding of [^{3}H]DHA occurs with negatively-charged glass fibre filters (GF/C) which were presoaked in buffer solution and is not prevented by presoaking the filters in 1 mg/ml BSA. Spurious binding to uncharged paper filters was eliminated after the addition of BSA to the presoak buffer. This type of binding does not occur with GF/C presoaked with 0.5% polyethylenimine (PEI). This example illustrates how the charge on the filter can lead to spurious binding, presumably as a result of ionic interactions between the ligand and the filter. This was substantially reduced by including the cationic PEI in the filter presoak buffer to reduce the negative charge on the glass filters as shown previously for the binding of a number of other tritiated ligands to various receptors in rat brain membrane preparations including adrenergic, dopaminergic, muscarinic, opiate, bradykinin and benzodiazepine receptors (Bruns et al., 1983). An excess of the unlabelled beta-adrenoceptor antagonist, propranolol with 0.5% PEI, further reduced the spurious binding to negligible levels as shown in Figure 2.5.

(Figure 2.5. near here)

This spurious binding has been previously observed in more purified membrane preparations by the some authors (Cuatrecasas and Hollenberg, 1975), but, without systematic investigation, may easily lead to erroneous conclusions, particularly with reference to the existence of secondary specific binding sites of low affinity (Cook and Bielkiewicz, 1985). The resultant distortion of the hyperbolic equilibrium binding curve has particular relevance to the determination of B_{max}; the number of binding sites may be severely overestimated under assay conditions which favour binding to both high and low affinity sites.

It is evident that the advantages of retaining all the specific binding sites of interest in the muscle homogenate may be negated by the inability to distinguish these sites from non-specific and spurious specific binding sites unless the latter two are substantially reduced or eliminated. The principal means of doing as is to select the appropriate filters, unlabelled competitive ligands and buffer solutions to effectively block or dilute the non-specific and spurious binding sites. Thus, the reduction of non-specific binding and the elimination of artificial specific binding of ligands to filters makes possible the quantitative resolution of ligand binding to receptors as shown in the comparison of data in Figure 2.4. and 2.6. (see also, Bambrick and Gordon, 1986 and 1987; Howlett and Gordon, 1986 and 1987).

(Figure 2.6. near here)

2.2.3.2. Normalization

Because limited sites are lost in the homogenate preparation, numbers of ligand binding sites can be normalized by a variety of muscle parameters to allow for comparisons between muscles which differ according to developmental stage. By normalizing [^{3}H]STX binding sites in neonatal rat muscles by muscle weight, protein and fibre diameter, developmental changes in Na channel density are revealed, despite concomitant developmental growth in the muscles (Table 2.1.). For example, when [^{3}H]STX binding is expressed relative to muscle wet weight or protein concentration, the number of binding sites in the muscle appears to plateau at 3 weeks of age to adult. By contrast, when binding site density is expressed relative to protein concentration, the number of binding sites continues to increase with age suggesting that increasing numbers of Na channel proteins are still being incorporated into muscle membranes after 3 weeks of age.

(Table 2.1. near here)

Because the recovery of ligand binding sites in homogenates is nearly 100%, these data are easily compared with literature values for factors not directly studied, such as the number of muscle fibres or nuclei (Harris, 1981) or the physiological properties of the membrane protein to which the ligand binds (Harris and Marshall, 1973; Pappone, 1980). Additional normalization factors which may be considered include other specific proteins of interest, muscle DNA content and mRNA content both in general and in terms of the specific mRNA which codes for the ligand binding site of interest (Cooperman et al., 1987; Witzemann et al., 1987). Along with the greater ease of homogenate preparation and minimal tissue requirement, the flexibility given by the wide choice of normalization factors is the main advantage of this method when compared to methods which use more purified membrane preparations. The latter preparations are extremely valuable in elucidating both the subcellular localization of binding sites during the processing of the receptor or ion channel proteins and for more detailed biochemical characterization of the proteins which is not possible with homogenate preparations.

Membrane preparation are potentially more attractive for determining binding site densities but there are a number of major problems which lead us to maintain that homogenate preparations are more suitable for quantitation of sites in muscles under conditions of plasticity. These problems are: 1) contamination of the most purified preparations with membranes other than the membranes of interest, as, for example, in striated muscle where contamination of sarcolemmal preparations with sarcotubular membranes is a particularly confounding problem (Barchi et al., 1979). 2) Variable yields of required membrane from preparation to preparation make it difficult to compare site densities form animal to animal, much less the densities obtained from different preparative techniques in different laboratories. This is particularly true for studies of developing muscles where the yield of membrane may vary considerably (Engel and Stonnington, 1974; Volpe et al., 1982; De Coster et al., 1985). A common solution is to ensure that a number of membrane proteins of known localization other than the one of interest are assayed as specific markers during purification. However, not only may the membranes of interest be differentially lost in purification when control and experimental muscles are compared, but the proteins which are used as markers are themselves changing. For example, the most common marker of sarcolemma, Na^+-K^+ ATPase, changes throughout neonatal life (Kjeldsen et al., 1982, 1984; Ward et al., 1987). Calcium-binding proteins of the sarcoplasmic reticular membranes are similarly unreliable measures as they are changed after experimental manipulations such as denervation (Lucas-Heron et al., 1986). 3) Prohibitive quantities of tissues are required for some membrane purifications, presenting particular problems when using small laboratory animals. It is not uncommon that many animals may be required for a single assay of membrane proteins in adult muscle and in small fetuses, that number may be as high as 1200 (Renaud et al., 1981).

2.2.3.3. Tissue Heterogeneity

The muscle homogenate preparation, by necessity, includes all muscle membranes (t-tubular membranes as well as sarcolemma) and the smooth muscle and endothelium of the blood vessels. Where the receptor or channel proteins being studied are present in more than one of these membranes, this complicates the interpretation of muscle homogenate binding. The affinity of receptors in these different membranes may either be similar or quite different. For example, the heterogeneity between the sodium channels in autonomic nerve endings and in the ventricular myocardium becomes evident in the appearance of two binding sites in cardiac homogenates (Catterall and Coppersmith, 1981). This possibility should be checked by eliminating the tissues other than the muscle of interest.

One method illustrated in Figure is to isolate the muscle of interest. Viable myocytes prepared by cell dispersion with collagenase treatment (Hunter, 1986) demonstrate the same affinity for [^{3}H]DHA as homogenate preparations and the number of sites determined in homogenates of either ventricular myocytes and ventricular muscles were the same.

(Figure 2.7. near here)

This approach may also be used where the ligand binding to intracellular membranes is a problem. By incubating whole cells in suspension, as in the case of dissociated myocytes (DePover et al., 1983; Buxton and Brunton, 1986), skeletal muscles, or cell cultures (Shainberg and Burstein, 1976; Frelin et al., 1981; Black and Hall, 1985), binding of the ligand is confined to the receptors or channels in the sarcolemma.

In summary, this paper shows how quantitative and reproducible assays of muscle receptors and ion channels can be carried out in muscle homogenates using radioligand binding techniques. A general framework for the development of effective binding methodologies in homogenates of skeletal, cardiac and smooth muscle has been presented using a variety of radioligands and particular problems inherent in the use of crude homogenates have been addressed. The advantages of using muscle homogenates include ease of preparation, negligible loss of binding sites during preparation and the relative ease of normalizing the data by muscle protein, weight, fibre diameter or other factors. Although whole muscle, dissociated muscle cell and purified muscle membrane preparations may also be used effectively in ligand binding studies, we feel that these techniques are often best employed as a second step after the less ambiguous muscle homogenate binding is described.

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Table 2.1. Normalization of $[^3H]$STX sites to sodium channels in developing rat triceps surae muscles.

Postnatal age	$[^3H]$STX specifically bound[1]			
Weeks	fmol/mg protein	fmol/mg muscle muscle weight	fmol/muscle	fmol/muscle surface area[2]
2	25 ± 1	4.1 ± 0.2	384 ± 1	22.5 ± 1.8
3	54 ± 3	7.4 ± 0.3	2005 ± 81	64.2 ± 2.8
4	60 ± 11	7.8 ± 2.5	3838 ± 1230	72.7 ± 11.8
7	59 ± 12	7.8 ± 1.0	10764 ± 1785	107.8 ± 18.2

[1] Values for $[^3H]$STX bound are X ± S.E.M. for 4-6 rat triceps surae muscles.

[2] The number of sites per unit volume of muscle multiplied by the volume:surface area ratio of a cylinder or r/2.

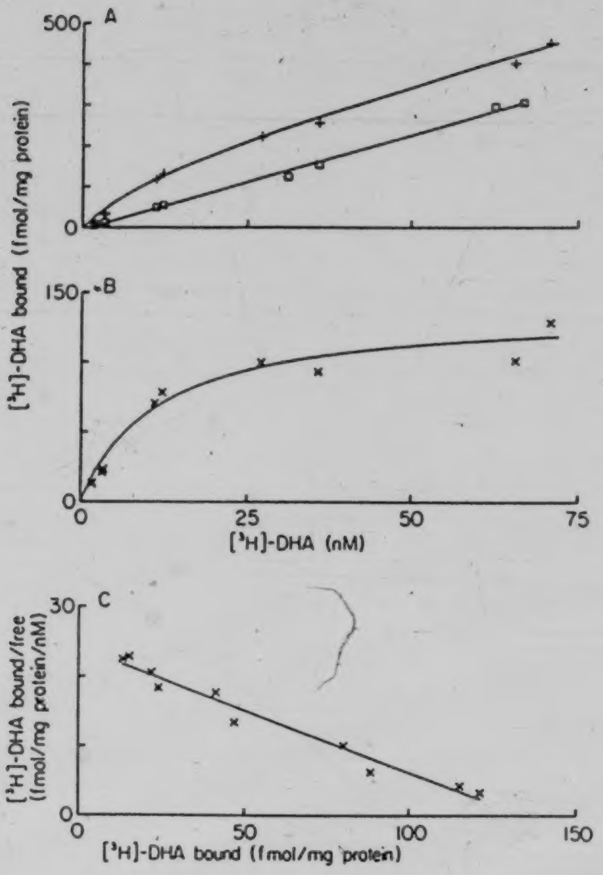

Figure 2.1. A) Equilibrium binding of [^{3}H]dihydroalprenolol to cardiac muscle homogenates prepared from the ventricles of a 3 month-old Dutch rabbit in the presence (non-specific, ☐) or absence (total, +) of propranolol. B) Subtraction of the non-specific binding from the total binding yields the specific binding (×) which is fitted, using least-squares criteria, with a hyperbolic curve of the form: $Y=B_{max} \cdot F/(F+K_d)$. C) B_{max} and K_d were also measured from a Scatchard plot obtained by replotting the data in B) where B_{max} is the maximum amount bound and the K_d is the affinity of binding. In this example, the K_d = 5.8 nM and the B_{max} = 130 fmol/mg protein.

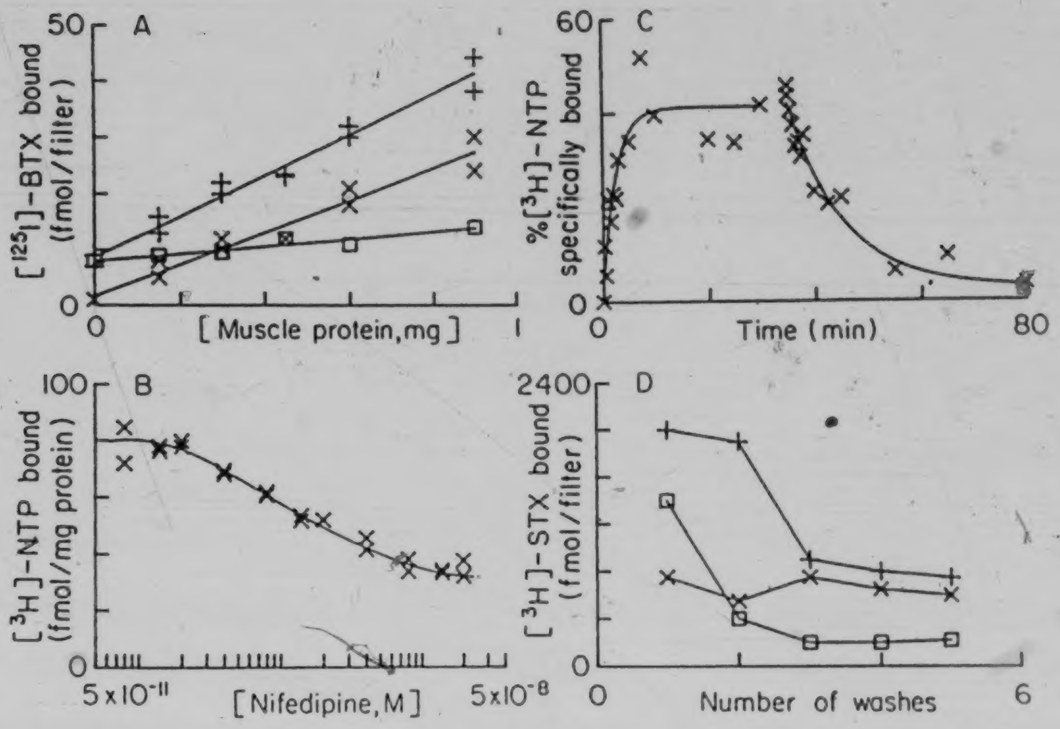

Figure 2.2. A), Total (+), non-specific (□) and specific (×) binding of [^{125}I]BTX to rat skeletal muscle homogenate showing that both the total and specific binding increase in a linear fashion with increasing concentrations of muscle protein. Note that a considerable amount of muscle protein. B) The specific binding of [^{3}H]NTP to normal hamster cardiac muscle homogenate is progressively displaced by increasing concentrations of unlabelled nifedipine. In this example the concentration of unlabelled drug producing 50% inhibition of specific binding (IC$_{50}$) = 1.47 nM and the corresponding affinity constant for the unlabelled drug (K$_i$) = 0.34 nM. Note

that the amount of [^{3}H]NTP binding remaining after full displacement is the non-specific binding at this ligand concentration. C) The [^{3}H]NTP specifically bound to dystrophic hamster heart increases with time until an equilibrium between the rates of drug association and dissociation has been established. In this example, the net rate of accumulation of the ligand-receptor complex is 0.306 min^{-1} and the rate constant for association is 1.084 min^{-1}nM^{-1}. At equilibrium a large excess of unlabelled drug was added, leading to dissociation of the bound drug. The dissociation rate constant is 0.053 min^{-1}. D) The total (+) and non-specific (□) binding of [^{3}H]STX to rat brain homogenates (brain homogenates were prepared as described in Baumgold et al., 1983) decreases with the number of filter washes in contrast to the specific (×) binding, which remains constant showing that increasing the number of filter washes is an effective way to reduce filter binding in this assay. Similar results were obtained with skeletal muscle homogenates.

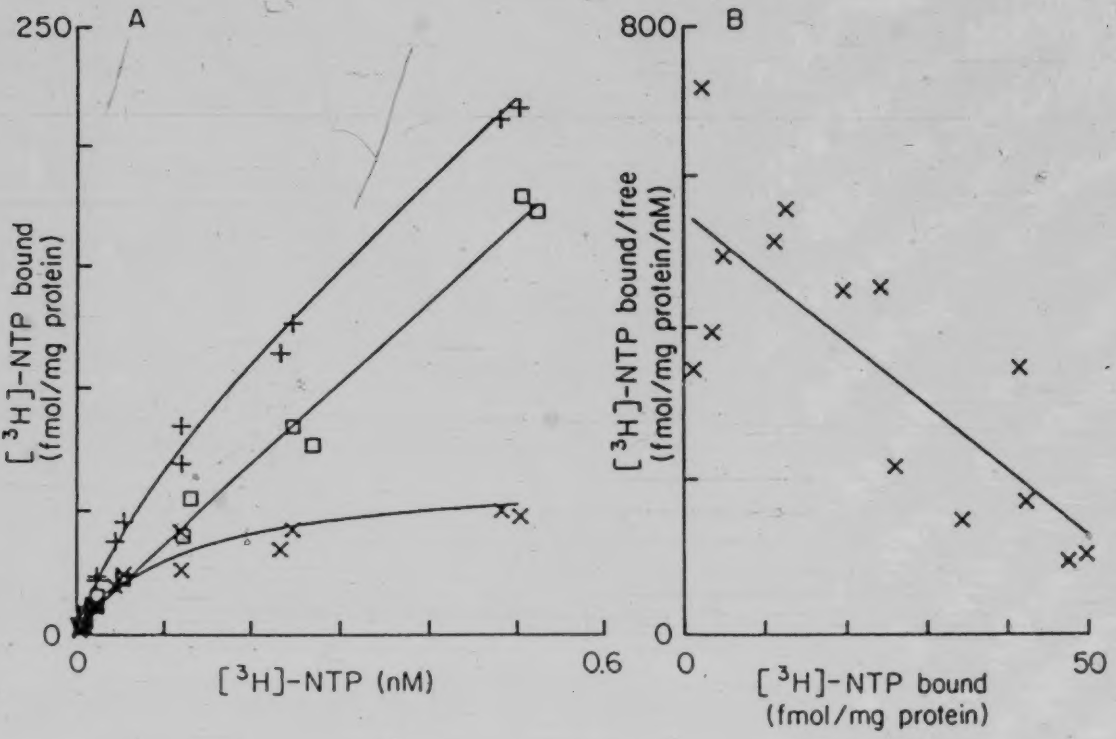

Figure 2.3. A) A large component of the total (+) [^{3}H]NTP binding to normal hamster cardiac muscle homogenates is non-specific (□) binding unless further procedures, which will be described later, are adopted; the specific (×) binding is significantly lower than the non-specific binding, particularly at saturation. B) The specific binding data replotted as a Scatchard plot illustrate the relatively poor fit of these data to a straight line (r = 0.7). In this example, the K_d = 0.12 nM and the B_{max} = 65 fmol/mg protein.

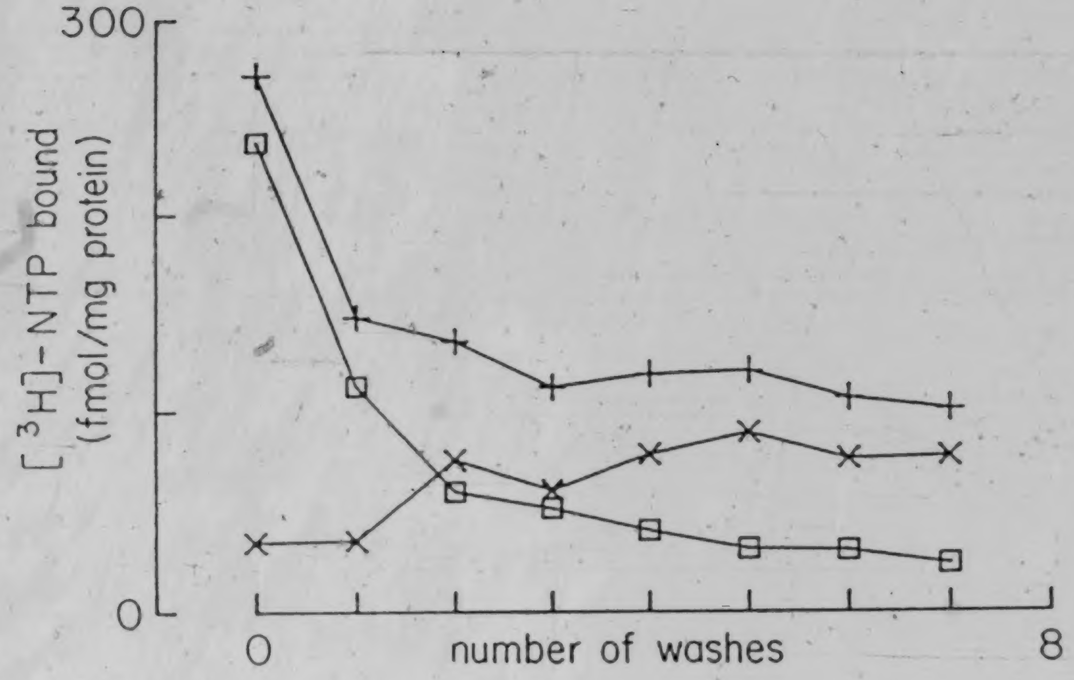

Figure 2.4. Total (+) and non-specific (□) binding of [^{3}H]NTP to the longitudinal smooth muscle of the guinea pig ileum decreases with an increasing number of 5 ml filter washes (50 mM Tris·HCl, pH 7.4) without affecting specific (x) binding.

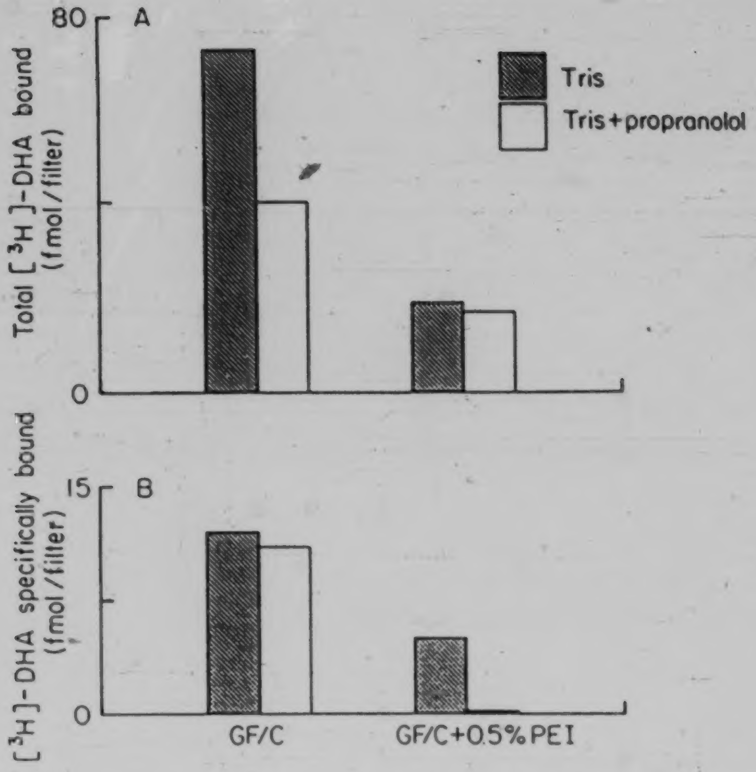

Figure 2.5. A substantial component of the A) total binding of [^{3}H]DHA to glass fiber filters (GF/C) in the absence of muscle homogenate is B) specific filter binding. Inclusion of 10^{-4} M propranolol in the presoak buffer substantially reduces non-specific filter binding, but does not appreciably reduce specific filter binding. Presoaking the filters in 0.5% PEI markedly reduces both specific and non-specific filter binding, particularly in the presence of propranolol.

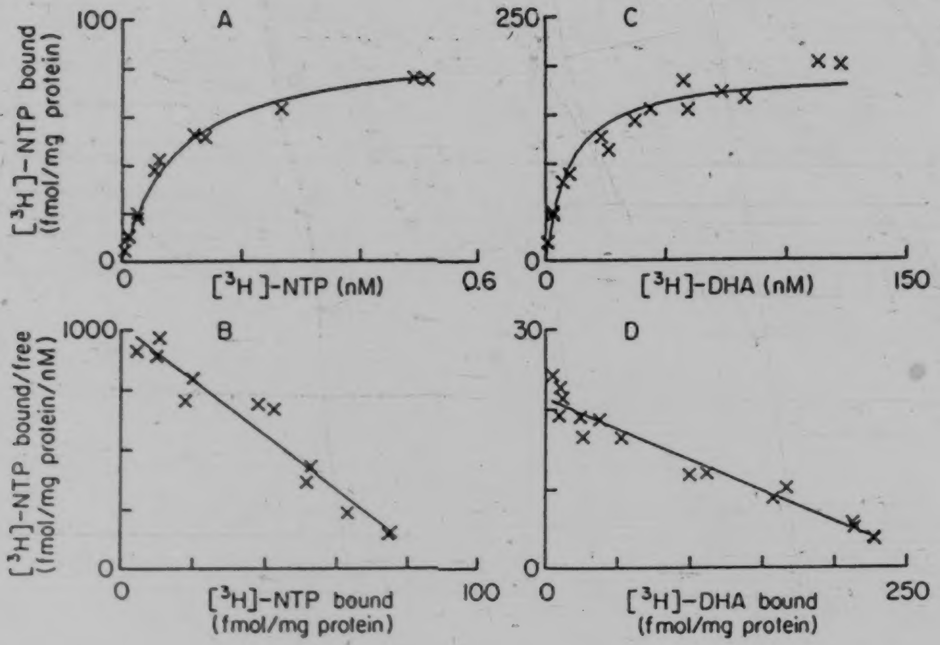

Figure 2.6. A) Specific [^{3}H]NTP binding to dystrophic hamster cardiac muscle increases as a function of the increasing concentration of free [^{3}H]NTP. These data are replotted as a Scatchard plot in B) showing that [^{3}H]NTP binds with high affinity and in a saturable manner to a single population of sites in cardiac muscle homogenates. In this example, the K_d = 0.09 nM and the B_{max} = 89 fmol/mg protein (r = 0.97). C) The binding of [^{3}H]DHA to heart homogenates from 3 week-old Dutch rabbits increases as a function of free [^{3}H]DHA. D) A Scatchard plot of the data shows binding to a single population of high affinity binding sites with a K_d= 14 nM and B_{max} = 210 fmol/mg protein (r = 0.85). Note that the B_{max} normalized per mg protein is substantially higher in the developing rabbit heart used in this example than in the adult rabbit heart illustrated in Figure 2.1.

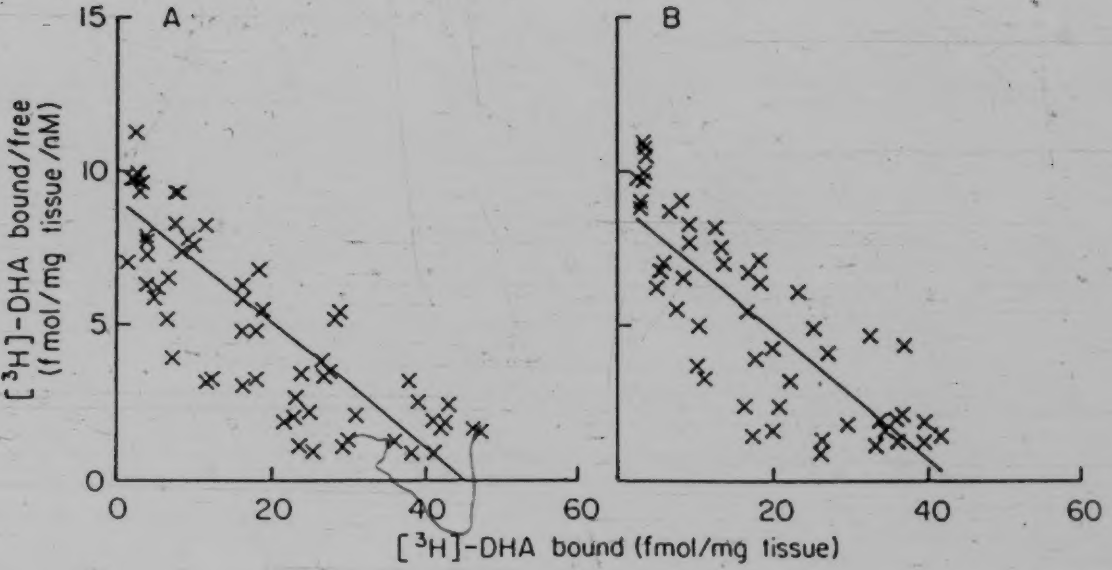

Figure 2.7. [^{3}H]DHA binds to a single population of high affinity binding sites in both A) homogenate preparations (n=8) and B) isolated myocytes (n=4) from 3 month-old Dutch rabbit ventricles. In these examples which illustrate pooled data from several different binding experiments in each case, K_d = 4.2 ± 0.4 and 4.1 ± 0.5 nM and B_{max} = 31.4 ± 4.5 and 34.5 ± 4.4 fmol/mg tissue for the homogenate and myocyte preparations, respectively, (X ± S.E.M.).

CHAPTER III. MYOCARDIAL INOTROPISM AND RADIOLIGAND BINDING OF 1,4-DIHYDROPYRIDINES IN DEVELOPING VENTRICULAR MUSCLES*

Z.-P. Feng, T. Gordon and W.F. Dryden

3.1. INTRODUCTION

In the adult heart, internal calcium (Ca) stores in the sarcoplasmic reticulum (SR) rather than external Ca provide activator Ca for myocardial contractility on a beat-to-beat basis (Fabiato and Fabiato, 1975; Fabiato and Fabiato, 1978). In the newborn heart on the other hand, external Ca is more likely, at least on a temporary basis, to be the major source of activator Ca prior to maturation of the SR (Nayler and Fassold, 1977; Nishioka et al., 1981; Maylie, 1982; George and Jarmakani, 1983; Seguchi et al., 1986; Pegg and Michalak, 1987; Michalak, 1988). The findings that Ca channel blockers are more effective in reducing muscle contraction in the neonatal heart muscles (Boucek et al., 1984; Artman et al.,

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* A similar version of this chapter has been submitted to Am. J. Physiol. for publication. The results of this chapter have been published in abstract form [Feng Z-P, Dryden WF and Gordon T. Radioligand binding and functional studies of calcium channels during postnatal development of rabbit ventricular muscles. FASEB J. 1988, 2:A372.].

1985) is consistent with this idea even though it has been reported that the inotropic effect of external Ca was small (Park et al., 1980; Jarmakani et al., 1982). These findings may be reconciled if the effectiveness of external Ca as the activator Ca were limited by the number and/or immaturity of the sarcolemmal Ca channels.

The present study was undertaken to examine the role of extracellular Ca in myocardial contractility in the developing heart by using the 1,4-dihydropyridine (DHP) derivatives to determine a) the number of myocardial Ca channels and their properties during postnatal development and b) the relative contribution of these channels to myocardial contractility in the maturing heart of rabbits. The dihydropyridines bind to L-type Ca channels and act as either agonists or antagonists to modulate the trans-sarcolemmal Ca currents (Lee and Tsien, 1983; Bean, 1984; Hess et al., 1984). The tritiated form of the Ca antagonist, [^{3}H]-nitrendipine, has been used to assay the number of Ca channels in the developing heart and the affinity of the DHP-binding site for nitrendipine while another antagonist, nifedipine, and the agonist, Bay K8644, have been used to examine the inotropic effects of external Ca. The results of these studies indicate that external Ca is the major source of activator Ca but that the inotropic effect of Ca in the newborn heart is limited by the small number of Ca channels and by their immature properties. The results have been reported in abstract form (Feng et al., 1988).

3.2. METHODS

The experiments were performed on 1-3 day-old (newborn), 20-22 day-old (juvenile) and 2-4 month-old (young adult) Dutch rabbits, of either sex. The rabbits were sacrificed by cervical dislocation and the heart muscle prepared for either radioligand binding or isometric contraction studies as described below.

3.2.1. [^{3}H]Nitrendipine equilibrium binding

3.2.1.1. Muscle preparations

Ventricles were dissected, weighed and homogenized in ice-cold HEPES solution (HEPES 20 mM, sucrose 250 mM, titrated to pH 7.8 with 1M Tris base) using a Brinkmann Polytron at half maximal speed for 2 x 10 s bursts. The whole homogenate was filtered through 4 layers of surgical gauze, diluted in 10 volumes (w/v) of HEPES solution, and either used immediately or kept at -70°C prior to equilibrium binding assay. An aliquot of homogenate was taken to determine protein concentration by the method of Lowry et al. (1951) with a range of bovine serum albumin standards.

3.2.1.2. Equilibrium binding assay

Tritiated nitrendipine ([^{3}H]NTP) (specific activity 85.9 Ci/mmol) was used to assay the number of Ca channels in ventricular homogenates as described in detail by Howlett and Gordon (1987). Briefly, duplicate assays of total and non-specific binding of ^{3}[H]-NTP to ventricular homogenates were carried out in the absence and presence of a large excess of unlabelled NTP (1 uM). The assays were initiated by adding about 500 ug ventricular homogenate protein to test tubes containing 0.005 - 1.0 nM [^{3}H]NTP, with or without 1 uM nifedipine in a final volume of 2 ml with 50 mM Tris·HCl solution (pH 7.4). After 90 min incubation at 20°C in light-protected conditions, 50 ul aliquots were removed from each tube for counting and subsequent calculation of the free [^{3}H]NTP concentration. The remainder was then filtered through Whatman GF/B filters with six washes of 5 ml ice cold 50 mM Tris·HCl using a Brandel cell harvestor (Model 24 RC Brandel, Inc., Gaithersberg, Maryland, USA). The radioactivity of washed filters and 50 ul aliquots was measured using a Beckman liquid scintillation counter with quench correction.

3.2.1.3. Data analyses

Specific NTP binding, obtained by subtracting non-specific from total binding (Figure 3.1A), was plotted as a function of free NTP and fitted, using least mean squares criteria, with a hyperbolic curve of the form $B=(B_{max}\cdot[F]/([F]+K_d)$, where B_{max} is the maximum number of specific binding sites, K_d is the dissociation constant, and B is the amount of ligand bound specifically at [F], the concentration of free ligand (Figure 3.1B). Values of B_{max} and K_d were also calculated by replotting the data as a Scatchard plot (Figure 3.1C), where the x intercept is the B_{max} and the slope of the fitted regression line is $-1/K_d$. The B_{max} and K_d values obtained from hyperbolic curves were always in good agreement with estimates of the same variables obtained from the Scatchard plots. The data were only acceptable where the regression lines were significant at the 1% level of confidence. The B_{max} and K_d values from individual experiments were expressed as arithmetic and geometric mean (± S.E.M.) respectively, for 4 - 8 preparations in each group.

Numbers of binding sites in muscle preparations were normalized by tissue weight rather than homogenate protein since we found that total protein per unit wet weight increased with development (Bambrick et al., 1988; Chapter II.).

(Figure 3.1. near here)

3.2.2. Isometric contractility

3.2.2.1. Muscle preparations and experimental procedure

Ventricular papillary muscles were isolated from rabbits in each of three age groups and mounted in 4ml tissue baths containing oxygenated Krebs-Henseleit solution (Composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 10; pH 7.4), maintained at 37°C. Muscles were attached to Grass isometric force displacement transducers (FTO3B), which were coupled to a Grass polygraph (Model 5) for amplification and paper recording. The muscles were stimulated via bipolar punctate platinum electrodes with 5 ms square-wave pulses of 2 x threshold voltage at a frequency of 1 Hz. The length of each tissue was adjusted to provide twitch tension equal to 70% of maximum. A period of at least 1 hour was allowed to elapse to ensure stable performance of the muscles.

3.2.2.2. Concentration-effect relationship

DHP derivatives, nifedipine (a Ca channel antagonist) and Bay K8644 (an agonist), were dissolved in polyethylene glycol-400 (PEG-400) and diluted with Krebs-Henseleit solution. PGE-400 alone had no effect on contractile function at the concentration employed (the maximum bath concentration of PGE-400 was about 0.02 mg/ml).

Either nifedipine or Bay K8644 was added incrementally to the tissue bath to obtain a cumulative concentration-effect relationship. Control tension was recorded at steady state before the drugs were added and 15 - 30 min after each drug addition when contractile tension had restabilized. Experiments were performed under light protected conditions since these drugs are photosensitive.

Cumulative concentration-effects of extracellular Ca on papillary muscles were examined in tissue baths which initially containing a low Ca Krebs-Henseleit solution (0.1 mM $CaCl_2$) before cumulative addition of higher concentrations of external Ca. The isometric contractile tension was allowed to equilibrate over 5 min interval before further addition of

$CaCl_2$ to the baths.

3.2.2.3. Data analyses

The contractile response of 5 - 9 papillary muscles to each concentration of DHP's or $CaCl_2$ were expressed as percentage of steady state tension obtained in the absence of the drugs or the initial tension obtained at 0.1 mM $CaCl_2$, respectively, and averaged as the arithmetic mean (±S.E.M.). The concentrations of nifedipine that reduced twitch tension to 50% of the control tension (IC_{50}) were expressed as geometric mean, i.e. the mean of the log values ± S.E. of the mean. The geometric mean concentration-effect curves of Bay K8644 or $CaCl_2$ to elicit responses of 10, 30, 50, 80 and 100 % of the maximal responses were normalized as described in detail by Carpenter (1986).

3.2.3. Statistics

Ducan's multiple range test was used for comparing multiple samples of unequal size (Walpole, 1974; Dowdy and Wearden, 1983). Differences were considered to be significant if the p value was less than 0.05.

3.2.4. Drugs and chemicals

All ingredients of Krebs-Henseleit solution were of A.C.S. quality and obtained from Fisher Scientific Co. (Canada). All other chemicals were purchased from Sigma

Chemical Co. (St. Louis, MO.) with the following exceptions. Aquasol and [^{3}H]NTP were obtained from New England Nuclear (Boston, MA.). Nifedipine and Bay K8644 were the gifts of Miles Laboratories, Inc.

3.3. RESULTS

3.3.1. [^{3}H]Nitrendipine equilibrium binding to ventricular homogenates

As shown by the hyperbolic curves of specific binding and the single lines fitted to the data in the Scatchard plots in Figure 3.2., specific [^{3}H]NTP binding to ventricular homogenates in newborn, juvenile and young adult rabbits was saturable and consistent with a single population of the binding sites (the putative Ca channel) in each age group studied. The numbers of [^{3}H]NTP binding sites (B_{max}) are low in newborn hearts but increase by a factor of 3 in the first 3 weeks of postnatal life to reach adult levels (Figure 3.2A). The slopes ($-1/K_d$) of the regression lines in Scatchard plots, shown in Figure 3.2B, do not change during development. Thus the affinity of [^{3}H]NTP for its binding site remains constant during postnatal development.

When the mean values (±S.E.M.) of B_{max} and K_d from 6-8 preparations in each age group are compared in Table 3.1. and 3.2. respectively, it is clear that the B_{max} in the newborn is significant lower than in the older groups ($p<0.05$) and that the K_d values are not different between any age group. These data indicate that the NTP binding site in Ca channels in the immature heart resembles that in the adult, but that the number of channels increases during early postnatal life.

(Figure 3.2. near here)

3.3.2. Inotropic effects of nifedipine and Bay K8644

Despite the neonatal increase in numbers of the Ca channel, nifedipine becomes less effective in blocking muscle contraction during the same period of development. As shown in Figure 3.3., the decrease in isometric tension by nifedipine, expressed as a percentage of the tension developed in the absence of the drug, is concentration-dependent at all ages, over a broad concentration range, as shown previously in adult rabbit papillary muscles (Boucek et al., 1984; Artman et al., 1985). The negative inotropic effect of nifedipine is much greater at 3 days than at 3 weeks and 3 months (see also Table 3.1.). In addition, the concentration of nifedipine which reduced the tension to 50% (IC_{50}) was greater in the older muscles than in the newborn (Table 3.2.).

(Figure 3.3., Table 3.1. and 3.2. near here)

The finding of reduced sensitivity of the contractile system to nifedipine in the mature heart, concurrent with an increase in number of [^{3}H]NTP binding sites, suggests that external Ca becomes relatively less important than intracellular stores for activator Ca in the maturing heart, even though Ca channels are still being incorporated into the membranes. Nevertheless, the DHP agonist, Bay K8644, was effective in increasing contractile tension in paced papillary muscles at all stages of postnatal development. The positive inotropic action of a range of Bay K8644 concentrations increased progressively from 3 days to 3 weeks, and to 3 months of age (Figure 3.4A and Table 3.1.). This progressive increase may reflect an alteration in Ca channel behavior since the sensitivity of the contractile response to Bay K8644 also increased (Figure 3.4B and Table 3.2.). This increase in sensitivity with age, seen as a progressive, parallel and leftward shift of the concentration-effect curves in Figure 3.4B, provides some evidence to suggest that the gating properties of the Ca channel in the neonatal ventricles are different from the adult.

(Figure 3.4. near here)

3.3.3. Inotropic effect of $[Ca]_o$

The positive inotropic effectiveness of external Ca on the contractile system is high at 3 weeks (Figure 3.5A), when the numbers of Ca channels and the inhibitory effect of nifedipine on the contractile system has reached adult levels. In contrast, external Ca is much less effective as a positive inotropic agent in the adult (Figure 3.5A), even though the sensitivity of the contractile system to external Ca increases at the same time (Figure 3.5B). This increase in sensitivity is seen as a shift to the left of the plot of increased tension to $[Ca]_o$ expressed relative to the maximum response as a function of $[Ca]_o$ and the significantly lower EC_{50} values in the adult (Table 3.2.). These data indicate that the dependency of the contraction on external Ca decreases as the heart matures.

(Figure 3.5. near here)

3.4. DISCUSSION

The major finding of this paper is that the positive inotropic effect of extracellular Ca diminishes during postnatal development of the rabbit heart, even though the number of Ca channels increases with age.

3.4.1. Number and properties of calcium channels

The number of $[^3H]$NTP binding sites in rabbit ventricles increases rapidly during the first 3 weeks of life and then remains at a constant level until young adulthood (Figure 3.2 and Table 3.1). This finding is consistent with the data obtained by Kazazoglou et al. (1983), who reported that the number of $[^3H]$NTP binding sites in purified membrane preparations of rat heart was low at birth and dramatically increased to an adult level within 7 - 10 days. The unchanged Ca channel density in isolated sarcolemmal vesicles from developing hearts from 2 weeks of age to adult, reported by Boucek et al. (1985), is probably accounted for by the fact that the largest increase occurs prior to 2 weeks of age.

The early increase in the putative Ca channel occurs concurrently with an incorporation of beta-adrenoceptors into the sarcolemma. In both cases, the number of sarcolemmal proteins increases by a factor of 3 (Feng et al., 1989a). Although the nature of control of synthesis and incorporation of these membrane proteins is not well understood, the finding that the developmental increase in these sarcolemmal proteins preceeds the maturation of the sympathetic innervation (Friedman et al., 1968) and intracellular Ca cycling systems (Hirakow et al., 1980; Hoerter et al., 1981; Maylie, 1982; George and Jarmakani, 1983) suggests that net incorporation may result from lack of down-regulatory mechanisms in the immature heart.

The finding that the affinity of the DHP site for [^{3}H]NTP ventricular homogenates remains constant during development, suggests that the properties of the NTP binding sites in the sarcolemmal Ca channels are mature in the neonatal heart. We find that K_d values of [^{3}H]NTP binding to ventricular homogenates are several orders of magnitude less (nM) than the IC_{50} (uM) or EC_{50} (uM) values for the negative or positive inotropic effects of nifedipine or Bay K8644, respectively. This is consistent with previous reports that the affinity of the channel in depolarized fragments from adult hearts is much higher than in polarized cells (Miller and Freedman, 1984; Schwartz and Triggle, 1984; Hamilton et al., 1987).

Even though the affinity of the NTP binding sites in the

Ca channel of the neonatal heart is the same as the adult Ca channel, comparison of the inotropic effect of the Ca channel agonist, Bay K8644, with the developmental increase in Ca channel number indicates that the gating properties of the Ca channels differ from the adult. In the adult, Bay K8644 increases the probability of the Ca channel opening (Kokubun and Reuter 1984; Sangninetti and Kass, 1984; Thomas et al., 1987). If Bay K8644 was as effective in opening the Ca channel in the neonatal heart, the developmental increase in the positive inotropic effect of the drug would be predicted to be of the same magnitude as the increase in the numbers of Ca channels. This was not the case as the inotropic effect of Bay K8644 increased by a factor of 2.2 from the newborn to the young adult heart in contrast to the 3 times increase in Ca channel numbers (Table 3.1.). These findings suggest that the newly synthesized and incorporated Ca channels are not fully opened by Bay K8644. and that the progressive increase in the effectiveness of Bay K8644 may be due to a maturation of the gating mechanism of the Ca channel during postnatal life. The increased sensitivity of the maturing heart to Bay K8644 is consistent with this idea and may be readily tested by direct measurements of Ca currents in the developing heart.

Although the nature of the postnatal change in the Ca channel is unknown, some possibilities include the differential development of subunits within the channel and/or the maturation of second messenger systems. The DHP-sensitive

Ca channel, like the nicotinic acetylcholine receptor and Na channels, is composed of five polypeptide subunits of which the alpha$_1$-subunit is the ion-conducting and voltage-sensing unit and the beta- and gamma-subunits are regulatory units (Campbell et al., 1988; Froehner, 1988). In the ACh receptor, for example, the fetal gamma-subunit is replaced by the epsilon-subunit with a subsequent increase in closing time of the ACh mediated ion channel (for review see Schuetze, 1987). An analogous developmental sequence of Ca channel subunits is possible where incorporation of the subunits into membrane at different times may influence the gating of the channel. In addition, modulation of Ca channel gating probably involves a cAMP dependent phosphorylation of the subunit (Hagiwara and Byerly, 1981; Rinaldi et al., 1982; Hagiwara, 1983; Reuter, 1983 and 1984; Hille, 1984) and there is good evidence that the basal myocardial adenylate cyclase activity increases progressively with postnatal age (Clark et al., 1980; Vulliemoz et al., 1984;).

3.4.2. Contribution of external Ca to myocardial contractility

Our finding that the negative inotropic effect of nifedipine on the contractile tension of paced papillary muscles in newborn hearts is greater than in the adult (Figure 3.3.) agrees in general with the reports by Artman et al. (1985) and Boucek et al. (1984 and 1985) and is indicative of a declining reliance of the maturing heart on [Ca]$_o$ despite a concurrent increase in numbers of sarcolemmal Ca channels.

Consistent with this, external Ca became progressively less inotropic with age (Figure 3.5A), although the sensitivity to the external Ca increased (Figure 3.5B). The declining effectiveness of $[Ca]_e$ in the mature heart is presumably due to the maturation of intracellular Ca stores in the SR (Maylie, 1982; George et al., 1984; Seguchi et al., 1986), and external Ca becomes the trigger for the relatively large amount of activator Ca released from the SR in the adult myocardium (Fabiato and Fabiato, 1978; Langer et al., 1982). The changing role of external Ca from activator Ca to trigger Ca is also evident from the similarity of the maximal inotropic responses of high $[Ca]_e$ and Bay K8644 in the adult (Table 3.1.). In addition, developmental changes in the post-rest potentiation in isolated rabbit heart muscles are also consistent with this altered role of external Ca (Boucek et al., 1987; Feng et al., 1989b; Chapter IV, V).

The increase in the sensitivity to $[Ca]_e$ in the adult muscles (see also Park et al., 1980; Jarmakani et al., 1982) may be explained by a concurrent increase in the sensitivity of the Ca trigger site for Ca induced Ca release from the SR. In addition, the increased sensitivity in adults suggests that less Ca from the extracellular space is required for this Ca induced Ca release mechanism than in immature muscles.

In summary, this study shows that the number of Ca channels, which are incorporated into the membrane of maturing hearts, increases after birth to reach adult levels. Alterations of the sensitivity to DHP agonist and antagonist with age indicate that these channels are likely to be immature with respect to their gating properties at the time of birth. Nevertheless, the channels appear to be effective in allowing external Ca to enter the cell, and to contribute to tension development in immature hearts. With time, the external Ca becomes relatively less effective as the internal stores mature, despite the concurrent increase in the number of Ca channels.

Acknowledgments. We are grateful to the Canadian Heart Foundation (to T.G.) for support of this work and to Dr. S. Howlett and Dr. G. Lopaschuk for their valuable comments on the manuscript.

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Table 3.1. Comparison of the maximal $[^3H]$NTP binding sites in ventricular homogenates, the maximal positive inotropic effects ($+T_{max}$) of Bay K8644 and $[Ca]_o$, and the negative inotropic effect ($-T_{max}$) of nifedipine on papillary muscles from different age groups.

Postnatal age (days)	$[^3H]$NTP B_{max} fmol/ mg tissue	Bay K8644 $+T_{max}$ (%)	Nifedipine (0.1 uM) $-T_{max}$ (%)	$[Ca]_o$ $+T_{max}$ (%)
1-3	6.1±0.9	81.8±21.2	17.7±4.5	460±57
20-22	19.5±1.7*	110.9±29.5	40.5±2.8*	530±74
80-120	20.1±1.8*	177.9±39.4*	39.5±3.3*	212±34*

Each value represents the arithmetic mean ±S.E.M. from 5-9 experiments. *, indicates significant difference from the values in 1-3 days group in each column ($p<0.05$).

Table 3.2. Comparison of the affinity of ventricular homogenates to [^{3}H]NTP and sensitivities of papillary muscles to Bay K8644, nifedipine and external Ca during postnatal development.

Postnatal age (days)	[^{3}H]NTP K_d (nM)	Bay K8644 EC_{50} (uM)	Nifedipine IC_{50} (uM)	$[Ca]_o$ EC_{50} (mM)
1-3	0.15 (0.12 - 0.18)	1.91 (1.02 - 3.55)	0.36 (0.18 - 0.72)	3.36 (2.51 - 3.69)
20-22	0.17 (0.14 - 0.21)	0.81 (0.54 - 1.23)	3.34* (2.36 - 4.71)	4.00 (3.66 - 4.39)
80-120	0.17 (0.15 - 0.19)	0.20* (0.12 - 0.34)	4.71* (3.11 - 7.03)	1.83* (1.52 - 2.20)

Values are expressed as the geometric mean, with the 5 and 95% confidence intervals given below in parentheses. EC_{50}, concentration that increased developed tension to 50% of control; IC_{50}, concentration that reduced developed tension to 50% of control. *, indicates significant difference from the values in 1-3 days group in each column ($p<0.05$). The data were obtained from 5-9 experiments.

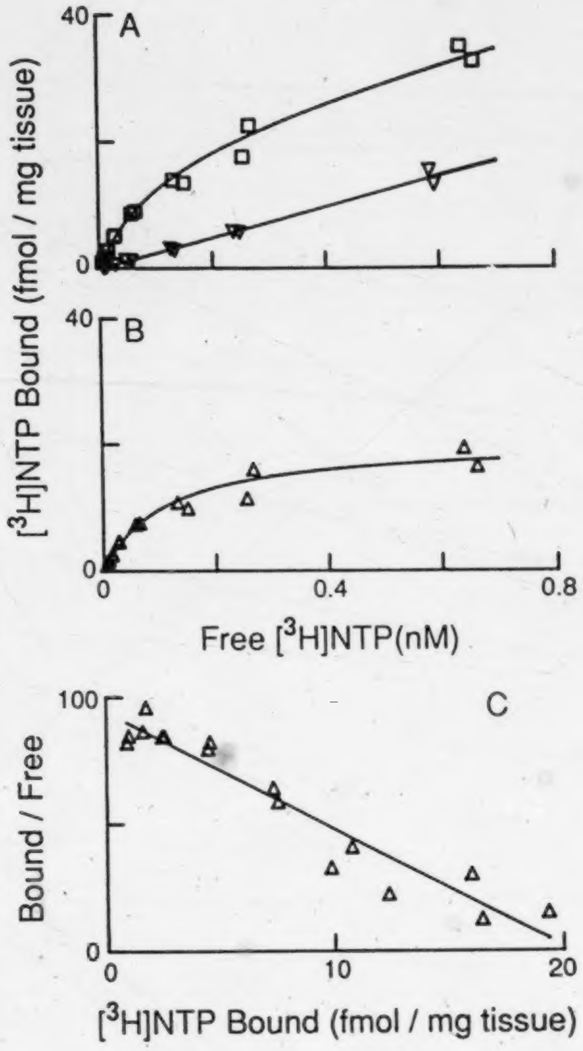

Figure 3.1. Equilibrium binding of [^{3}H]NTP to adult ventricular muscle homogenates. A), Total (□) and non-specific binding (▽) of [^{3}H]NTP to the homogenates in the absence or presence of 1x10^{-6} M nifedipine. The curve fitted

to the total binding data is the sum of the line fitted to the non-specific data and the hyperbola fitted to the specific binding data in B using least-squares criteria (Hartley, 1961). B), The specific [^{3}H]NTP bound (△) was obtained by substracting the non-specific component from the total [^{3}H]NTP bound in A. The points were fitted, using least-squares criteria, with a hyperbolic curve of the form:

$$Y = \frac{(P_1 \cdot x)}{(P_2 + x)}$$

where $P_1 = B_{max}$ and $P_2 = K_d$. C), Scatchard polt obtained by replotting the specific binding data. The intercept on the X axis is the B_{max} and the slope of the regression line is $-1/K_d$. In this experiment, B_{max} is 20.8 fmol/mg tissue·nM, K_d is 0.11 nM and the slope (± S.E.) of the regression line is −9.2±0.73.

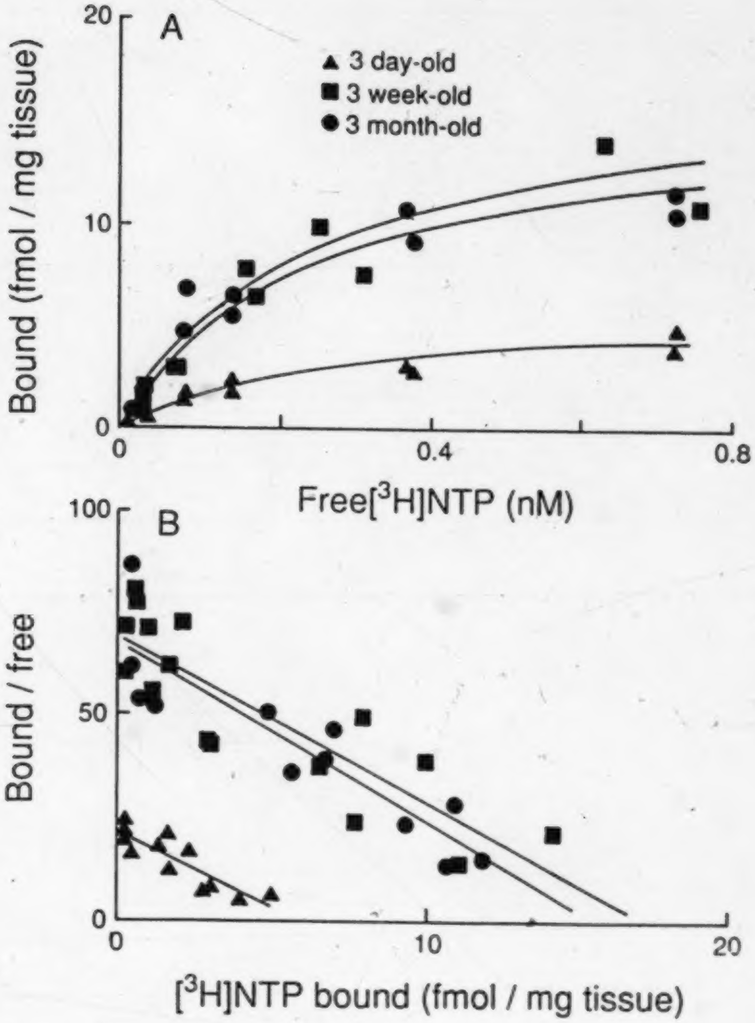

Figure 3.2. Specific [^{3}H]NTP binding to ventricular homogenates from sample individual experiments at different ages. Data were fitted using least-squares criteria either with hyperbolic curves (A) or with linear regression lines in Scatchard plots (B) as described in methods. (See Table 3.1. and 3.2. for mean ± S.E.M. values).

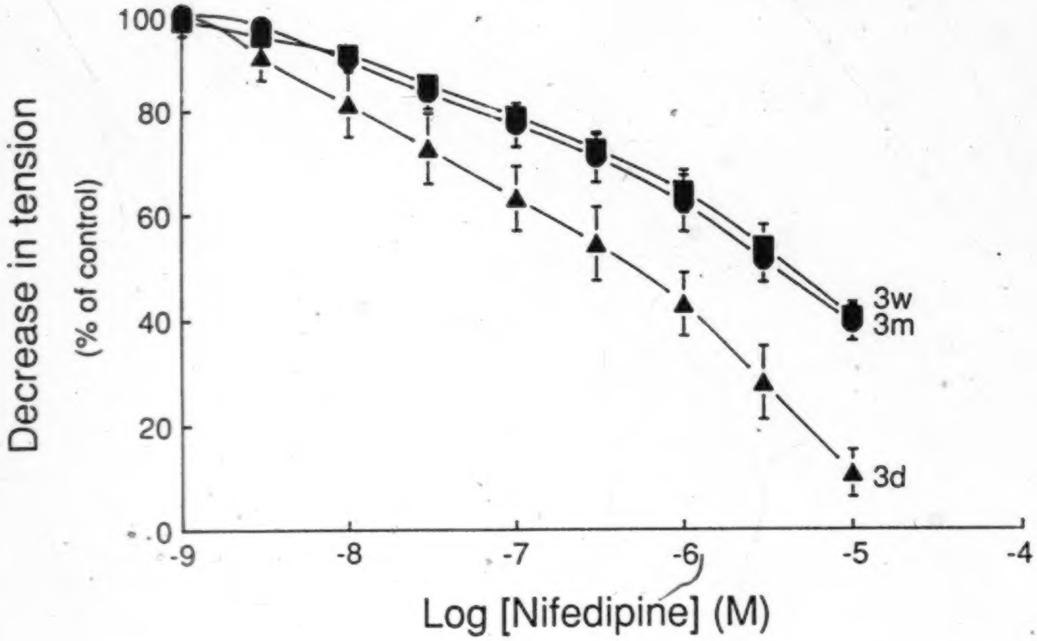

Figure 3.3. Concentration-effect curves of the negative inotropic response of developing papillary muscles to nifedipine. The data were plotted as the percentage decrease in tension of the control tension which was obtained in the absence of nifedipine. The nifedipine induced inhibition on the developed tension at each given concentration of the drug in the newborn (▲) is greater than that in juvenile (■), in which the nifedipine effect is similar to that in the adult muscles (●). Each point represents X ± S.E.M. from 6 - 9 experiments.

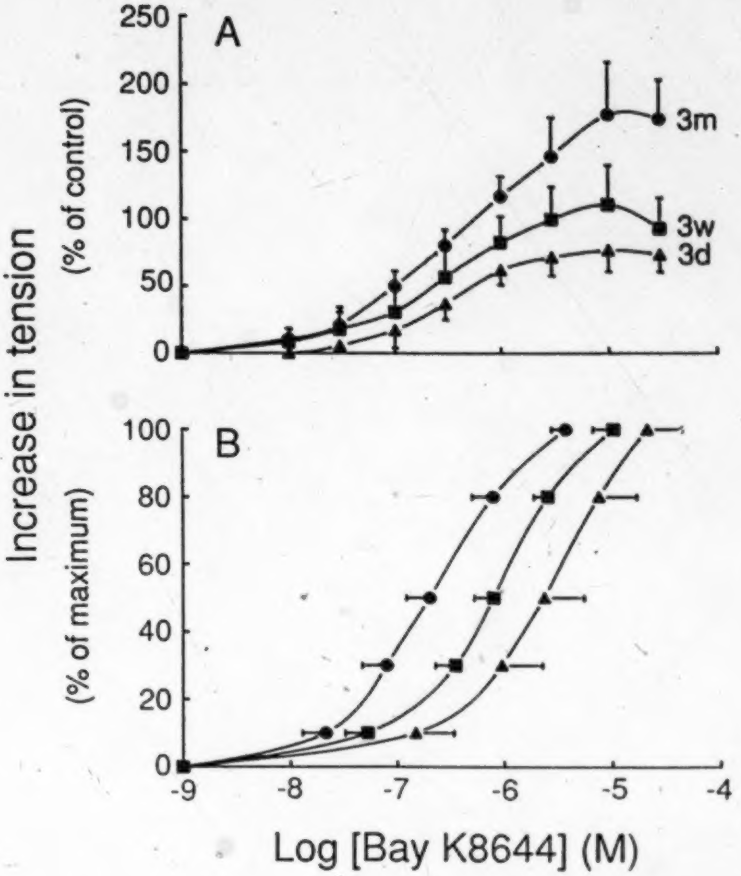

Figure 3.4. The positive inotropic effects of Bay K8644 on papillary muscles from different age groups. A) The arithmetic mean (± S.E.) of maximal percentage increase in tension in the presence of Bay K8644 was expressed as a percentage of the control tension recorded in the absence of Bay K8644, and found to increase progressively with the postnatal age. B) The same data are replotted as geometric mean (± S.E.) of the concentration for given response. The concentration-effect curves shift in parallel to the left with age. The data were obtained from 5 - 9 experiments.

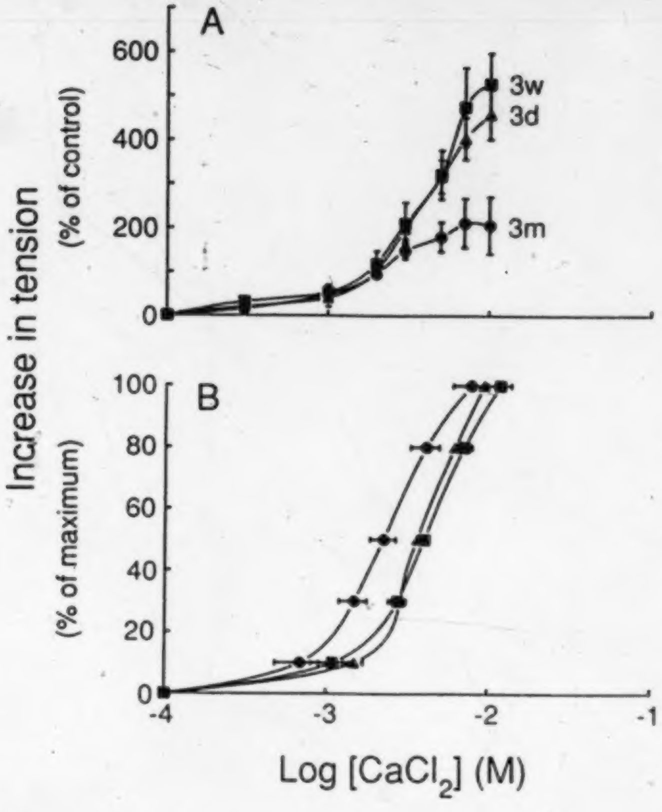

Figure 3.5. A) The positive inotropic effect of extracellular Ca was expressed as the percentage increase in twitch tension at 1.0 Hz of stimulation frequency above the control tension and plotted as arithmetic mean (± S.E.) for 6 - 9 papillary muscles as a function of extracellular Ca concentration. The positive inotropic effect of extracellular Ca decreased progressively with age as shown by a decline in the maximal percentage increase in twitch tension over the control tension at 10^{-4} M Ca. B) The same data were expressed as a percentage of the maximum response and the geometric mean (± S.E.) of each concentration computed for any given response. There is a significant shift of the curve to the left for muscles at 3 months from muscles at 3 days.

CHAPTER IV. EXCITATION-CONTRACTION COUPLING IN DEVELOPING VENTRICULAR MUSCLES*

Z.-P. Feng and T. Gordon

4.1. INTRODUCTION

The sarcoplasmic reticulum (SR), the major site of sequestration and release of intracellular Ca in the adult mammalian heart, is the primary source of activator Ca for myocardial contraction, although extracellular Ca via sarcolemmal Ca channels is important for triggering release of intracellular Ca from the SR and for maintaining intracellular Ca stores. Free intracellular Ca declines during relaxation as Ca is actively taken up by the SR and extruded from the cells primarily via the Na/Ca exchanger and the ATP dependent Ca pump (reviewed by Dhalla et al., 1982).

A number of studies have provided evidence that the relative contribution of extracellular Ca to myocardial contraction is greater than intracellular Ca in the newborn heart, prior to the maturation of the SR. Fabiato and Fabiato (1978) showed that Ca failed to induce Ca release from skinned ventricular cells from neonatal rat hearts suggesting that the intracellular compartment was poorly developed. Caffeine induced inotropism is less in newborn hearts (Jourdon et al., 1981). Morphological evidence support these physiological findings in demonstrating a poorly developed intracellular membrane system in the heart of newborn animals (mouse: Ishikawa and Yamada, 1976; rabbit: Page and Buecker, 1981; cat: Maylie, 1982). The fetal heart is devoid of T-tubules and the SR system is very sparse at birth (Penefsky, 1974a and 1974b). The inotropic effectiveness of external Ca and the Ca channel agonist, Bay K8644 in muscles from newborn rabbit hearts showed that extracellular Ca contributes a significant proportion of Ca to steady state force at this stage, even though the number of Ca channels has not yet attained adult levels (Feng et al., 1988; Chapter III). During neonatal maturation of the heart, external Ca contributes progressively less Ca to steady state force (Maylie, 1982; Boucek et al. 1984; George and Jarmakani, 1983; Feng et al., 1988).

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* The results of this chapter have been published in abstract form [Feng Z-P, Gordon T and Dryden WF. Do ryanodine insensitive intracellular Ca stores exist in the newborn rabbit papillary muscle? Can. Fed. Biol. Soc. 1989, #565].

In this study, we have examined the effects of stimulation frequency and external Ca on steady state and post-rest contractions of ventricular muscle to further evaluate the acquisition of mature excitation-contraction (E-C) coupling in cardiac muscle during postnatal development. Examination of post-rest contractions during interruption of steady state contractions at a constant priming frequency provides a powerful experimental tool for determining the distribution of Ca in the heart and the relative contributions of extracellular and intracellular Ca to E-C coupling because Ca distribution between extracellular and intracellular compartments is dynamically altered in mature muscles (Edman and Johannsson, 1976; Wohlfart, 1979; Bers, 1985; Schouten et al., 1987). The change in post-rest contractile force with interstimulation interval namely, mechanical restitution, is considered to reflect the restoration of releasable Ca for contraction (Bass, 1975; Hoffman et al., 1975), on the assumption that the post-rest contraction is a direct function of releasable Ca (Wood et al., 1969; Fabiato, 1982; Allen and Kurihara, 1980). At very short intervals (less than the interval at priming stimulation frequency), the amount of releasable Ca is compromised so that the post-rest contraction is less than steady state force (Hoffman et al., 1956; Antoni et al., 1969), but increases as a function of rest intervals at longer interval. At very long intervals (longer than 100 seconds), Ca in the release compartment of the SR declines as Ca exchanges with extracellular Ca via Na/Ca exchanger and to a lesser extent the Ca pump (Sutko et al., 1983; Schouten et al., 1987). The post-rest contractions are readily influenced by modulating exchange of Ca between extracellular and intracellular spaces by priming frequency, extracellular Ca concentration and interposition of extrasystoles via Ca channels and Na/Ca exchanger (Schouten et al., 1987). This dynamic alterations in force as a function of rest intervals con only occur if there are functional intracellular Ca compartments, so that examination of mechanical restitution during postnatal development provides a means of determining the presence and capacity of the functional Ca stores in the SR.

Even though there is evidence that the SR content is very low in neonatal heart muscles, purified SR from neonates have been shown to contain Ca binding proteins and ryanodine binding sites, the putative Ca release site in junctional SR (Pegg and Michalak, 1987, Michalak, 1988). These ryanodine binding sites defer from the adult in that there are high and low affinity sites. We have addressed the question of their functionality by determining whether contraction is sensitive to ryanodine in immature heart muscles.

4.2. METHODS

4.2.1. Isolated isometric contracting papillary muscles

The experiments were carried out using Dutch rabbits, of either sex, at 1-3 days (newborn), 20-22 days (immature) or 2-4 months (adult) of postnatal age. Rabbits were sacrificed by cervical dislocation, and each heart was rapidly removed and placed in Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.8, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10; pH 7.4) which was kept on ice and gassed with 95% O_2 and 5% CO_2. Ventricular papillary muscles were dissected free and mounted in 4 ml tissue baths containing oxygenated

Krabs-Henseleit solution maintained at 37°C. Muscles were attached to Grass isometric force displacement transducers (FT03B), which were coupled to a Grass polygraph (model 5) for amplification and paper recording. The muscles were stimulated via bipolar punctate platinum electrodes with 5 msec square-wave pulses of 2 x threshold voltage at a frequency of 1 Hz. The length of each tissue was adjusted to provide twitch tension equal to 70% of maximum. A period of at least 1 hour was allowed to elapse to ensure stable performance of the muscles.

4.2.2. Steady state contraction

FORCE-FREQUENCY RELATIONSHIP Rate of electrical stimulation on isometrically contracting papillary muscles from each age group at $[Ca]_0$ 2.8 mM, was increased stepwise from 0.1 to 5 Hz. The new steady state force reached after changing each stimulation frequency was measured at 3 min. Force-frequency curves were plotted as semi-log plots in which new steady state force was normalized by the maximum force.

4.2.3. Mechanical restitution

4.2.3.1. Experimental procedure

A digitimer (Devices) was used to control the timing of stimulation of 4 papillary muscles synchronously. By setting the clock frequency at > 10 minutes, one square wave pulse triggered a function generator (3310A) which was set to provide square wave pulses at the priming frequency of 0.2 - 2.2 Hz. These in turn triggered 4 separate Grass stimulators (SD9) to provide stimulus pulses to the muscles synchronously. A rest interval greater than the priming frequency (> 0.5 second) was interposed by delaying the trigger pulse from the digitimer by the required interval. A rest interval less than the priming frequency (< 0.5 second) was achieved by interrupting the frequency train with a 1 - 8 square wave pulses from a Grass stimulator (SD9) which were controlled manually by a switch control. The duration of these short intervals was therefore were variable but were measured by monitoring the pulse trains on a storage oscilloscope (type 564) and measuring the interposed interval directly. For intervals longer than 60 seconds, the function generator was switched off manually and the duration of the rest intervals were controlled by second timer.

4.2.3.2. Post-rest contraction

Papillary muscles from each age group was paced at a constant priming frequency of 1 Hz and the experiment commenced when a constant steady state contractile force ($F_{s.s.}$) was reached. The contractions in response to the priming frequency was interrupted by interposed rest intervals which were varied from 0.2 to 900 s. Force of the first beat after reinitiation of the priming stimulation, the post-rest force (F_1) was normalized by the steady state force ($F_1/F_{s.s}$)

and plotted as a function of the interposed rest interval, i.e. mechanical restitution curve. Post-rest contractions were examined at different priming frequencies and concentrations of extracellular Ca.

Extrasystoles in which a single pulse was interposed during steady state firing at 1.0 Hz. The priming frequency was terminated by a varied number of extrasystoles at 0.2 s intervals and the last extrasystole given was followed by varied rest intervals. Post-rest force after each rest interval was measured subsequently.

4.2.4. Effects of ryanodine

Effects of ryanodine, an alkaloid that specifically blocks the release of Ca from the SR (Sutko and Willerson, 1980; Sutko and Kenyo, 1983) were examined. Cumulative ryanodine dose-response studies were not performed in the present study since significant time-dependent effects of ryanodine were reported on myocardial contraction (George and Jarmakani, 1983, Boucek et al., 1987). Effects of ryanodine on $F_{s.s}$ and F_1 developed at 1 Hz of priming frequency and $[Ca]_o$ 2.8 mM were studied initially with a low concentration (0.1 nM) of ryanodine. Concentration of the drug was increased cumulatively until F_1 was reduced with minimal inhibition of $F_{s.s}$.

4.2.5. STATISTICAL ANALYSES

Results were expressed as the arithmetic means (± S.E.M.). Comparisons between each age group were performed using Duncan multiple range test. The probability level of significant difference was $p < 0.05$.

4.3. RESULTS

4.3.1. Steady state force

Consistent with many previous observations (Koch-Weser and Blinks, 1963; Shelburne et al., 1967; McCans et al., 1974), steady contractile force in adult papillary muscles increases with rate of stimulation. As shown in a typical example in Figure 4.1A, a change in frequency results in a progressively increase in force to a steady state level over a 10-fold range in frequency. Results from 11 adult animals are summarized in Figure 4.1B and compared with the force-frequency relations in immature muscles from neonatal hearts. The immature muscles show a similar force-frequency relationship to mature muscles except over a higher frequency range. As the heart matures, the curves shift to the left with age so that the inotropic effect of stimulation is seen at progressively lower frequencies. The high stimulation frequencies required in immature muscles are consistent with our previous results showing a progressive increase in the inotropic effectiveness of Bay K8644 and increase in Ca channel density (see Chapter III; Feng et al., 1988).

(Figure 4.1 near here)

One Hz was selected as the priming frequency in all subsequent studies of post-rest contractions since this is the frequency at which muscles at each age group develop 35-85% of their maximum force and at which Ca has sufficient time to move from the uptake to the release compartment of the adult SR (Edman & Johannsson, 1976; Wohlfart, 1979).

4.3.2. Post-rest contractions

4.3.2.1. Extrasystole

The response of post-rest contractions in immature papillary muscles provides evidence that Ca stores are present, although limited in capacity, are present. As illustrated in Figure 4.2, an extrasystole which is imposed during steady state contractions at a priming frequency of 1 Hz, is small relative to the steady state force in neonatal muscles as it is in mature muscles. Since force is a function of the releasable Ca, force depression of an extrasystole in adult muscle is accounted for by the insufficient time period for Ca to move from storage sites to release sites in the SR (Edman and Johannsson, 1976; Wohlfart, 1979 and 1982; Schouten et al., 1987). Since a similar depression occurs in the neonatal muscle, it is likely that activator Ca is, at least in part, released from an internal store. This store may be replenished by external Ca as shown by the potentiation of the second contraction following the extrasystole where 1 second interval is sufficient to allow replenishment of the release sites.

(Figure 4.2 near here)

The dynamic distribution of Ca between extracellular and intracellular compartments of immature muscles was examined in more detail by interrupting steady state contractions with progressively longer rest intervals (example as shown in Figure 4.3) and manipulating the exchange of intracellular with extracellular Ca by varying priming frequency, external Ca concentration and interposition of extrasystolic contractions (Figure 4.4).

(Figure 4.3 near here)

4.3.2.2. Mechanical restitution

In the adult, the mechanical restitution curve shows an increase to a peak which does not exceed the steady state level of force at a priming frequency of 1 Hz. Above the priming frequency interval, the post-rest contraction is progressively depressed (Figure 4.3B). Increase in the priming frequency increases the amplitude of the post-rest contraction at every internal but the increase is greatest between 1 and 100 s rest intervals (Figure 4.4A). This potentiation is consistent with an increased loading of internal stores 1) via the Ca channels which is seen as an increase in the peak force at 1 s, and 2) via the Na/Ca exchanger at intervals between 1 and 100 s (Schouten et al., 1987). Increasing external $[Ca]_o$ has very similar effects as shown by the dramatic potentiation in 10 mM $[Ca]_o$ (Figure 4.4B). Ca influx may be selectively increased via the Ca channel by interposing extrasystolic beats as illustrated in Figure 4.4C where the peak force is greatly elevated by increasing the number of extrasystoles. These do not affect post-rest contractions at longer intervals as they are unlikely to affect Na/Ca exchanger.

(Figure 4.4 near here)

The relative effect of raising priming frequency, extracellular Ca concentration, and number of interposing extrasystoles in the adult are shown in Figure 4.5A-C by replotting the data with the same Y axis for comparison. Developmental changes in E-C coupling and the relative contribution of internal and external Ca in immature and mature muscles are discerned by comparing the mechanical restitution curves in the neonatal papillary muscles under the same conditions of loading of intracellular Ca stores (Figure 4.5D-F).

The mechanical restitution curve, at 1 Hz priming frequency and 2.8 mM Ca in the newborn muscle is very similar to the adult muscle (compare filled circles in all the graphs in Figure 4.5). The finding that amplitude of post-rest contractions depends on the duration of the rest interval shows that there are functional intracellular Ca stores which contribute to force development in the immature muscle after birth. Further, potentiation of post-rest contractions by raising the priming frequency provides supportive evidence for the ability of intracellular SR stores to sequester and release Ca in the neonate. However, quantitative comparisons of the effects of priming frequency, high $[Ca]_o$ and extrasystoles reveal major differences in the dynamic distribution of Ca between extracellular and intracellular stores. 2Hz priming frequency is twice as effective in post-rest potentiation in immature muscles than adult (Figure 4.5A, D) which is consistent with the relatively greater contribution of external Ca to contraction in immature muscles. Thus finding also shows that Ca stores may be increased in the immature muscle via the Ca channels and Na/Ca exchanger.

Post-rest contractions at intervals between 1 and 100 s are significantly more potentiated by high extracellular Ca in the newborn (Figure 4.5B, E), which suggests that the Na/Ca exchanger in the immature heart is responsible for a greater proportion of sarcolemmal Ca influx in the newborn muscle than in the adult muscle. This is quite consistent with the high levels of Na/K ATPase activity described in immature hearts and the subsequent decline during postnatal development (see discussion section).

Extrasystoles were ineffective in potentiating post-rest contractions in the immature muscles in dramatic contrast to adult muscles (Figure 4.5C, F). This finding is consistent with the lower Ca channel density and limited gating characteristics in the immature muscles described previously

(Feng et al., 1988; Chapter III). Thus, although intracellular Ca stores may be progressively loaded by raising priming frequency or high external Ca, dynamic alteration of Ca influx by 4 - 8 extra beats is ineffective in the immature heart.

(Figure 4.5 near here)

Thus, high extracellular Ca is more effective in loading intracellular stores during steady state contractions in neonatal muscles 1) via Ca channels, despite the lower Ca channel density, and 2) via the Na/Ca exchanger. This is further illustrated by the later developmental changes in the more mature muscles at 3 weeks of age as shown in Figure 4.6. As Ca channel density increases during the first 3 weeks of age (Feng et al., 1988; Chapter III), high priming frequency (Figure 4.6A) and high $[Ca]_o$ (Figure 4.6B) are even more effective in potentiating the post-rest contractions at intervals between 1 and 100 s. Nevertheless by adulthood, external Ca is relatively ineffective in loading intracellular stores and there is little post-rest potentiation even at high frequency or $[Ca]_o$. This change coincides with a decline in activity of the Na/K pump (Akera, 1972; Marsh, 1981).

(Figure 4.6 near here)

4.3.3. Effects of ryanodine

Post-rest contractions in the immature muscles of the newborn rabbit are insensitive to ryanodine at concentrations of 0.3 nM to 0.3 uM at all rest intervals (Figure 4.7A, only

3 uM is shown) in contrast to more mature muscles in the 3 week old neonate and the adult muscles whose post-rest contractions are depressed at every interval by 0.3 uM ryanodine (see Figure 4.7C). Even in the more mature muscles, contractile force after intervals of 0.2 - 1 s was relatively insensitive to ryanodine. Concentrations of 10 nM ryanodine reduced post-rest contractions at longer intervals (Figure 4.7B, C). The finding that the amplitude of post-rest contractions in muscles from the newborn as a function of rest interval but that the contractions are ryanodine insensitive indicates that internal Ca stores contribute activator Ca to contraction but that the release sites, although they are present and bind ryanodine (Michalak, 1988) are immature in the sense of being functionally insensitive to ryanodine blockade. The finding that the contractions at intervals of less than 1 s is relatively ryanodine insensitive at all ages suggests that extracellular Ca contributes directly to activation of contractile proteins when the rest interval is too short for Ca to move from uptake to release compartments in the SR.

(Figure 4.7 near here)

4.4. DISCUSSION

The major findings of this paper are that intracellular Ca does contribute to E-C coupling in immature muscles of the newborn rabbit at a time when extracellular Ca is the major source of activator Ca, the SR is relatively poorly developed and internal Ca release sites are ryanodine-insensitive. Extracellular Ca, via Ca channels and Na/Ca exchanger contributes both directly to activator Ca and indirectly by loading the internal stores in the SR in the immature muscle, but the relative contribution of extracellular Ca declines as the SR storage capacity increases with age.

The SR is initially sparse in neonatal muscles. It is derived from the endoplasmic reticulum and proliferates postnatally (Jarmakani et al., 1984) in parallel with the contractile elements (Penefsky, 1983). At this time, steady state contractile force is highly dependent on external Ca, even though the sarcolemmal Ca channel density is still submaximal and gating properties of the channels is not fully mature (Feng et al., 1988; Chapter III). Nevertheless, at least a part of this external Ca serves to load internal Ca stores for contraction because post-rest contractions are dependent on both Ca and rest interval (Figure 4.4).

Even though, the T-tubular system is absent and the SR sparse, biochemical analysis of fetal SR indicates that Ca binding proteins are present in the immature SR and the membranes contain high proportions of ryanodine binding sites, the putative Ca release site (Michalak, 1988; Pegg and Michalak, 1987). Our physiological analysis of post-rest contractions provides convincing evidence that these internal stores of Ca participate in E-C coupling and that they may be loaded from the external Ca compartment via the Ca channel and Na/Ca exchanger. Firstly the amplitude of post-rest contractions is dependent on the duration of the rest interval in the immature muscles which is similar to that in the adult muscles. At intervals less than 1 second, the extrasystolic beats are depressed in the immature muscles as they are in the mature ones. This is consistent with insufficient time to move Ca from an internal uptake to a release compartment (Kaufmann et al., 1974; Morad and Goldman, 1973; Edman and Johannsson, 1976; Wohlfart, 1979; Schouton et al., 1987). Nevertheless, the following beat is potentiated in all ages (Figure 4.2). Following rest intervals greater than priming frequency, the amplitude of the post-rest contraction decreases in immature and mature muscles since sufficient time for Ca to be extruded from cells and Ca in release compartment to decline. These dynamic alterations in force as a function of rest intervals cannot occur in the absence of a functional intracellular Ca storage compartment, and thus our findings provide evidence that intracellular Ca stores do contribute activator Ca to contraction in immature muscles.

Exchange of Ca between extracellular and intracellular Ca compartments occurs through voltage-dependent Ca channels, the Na/Ca exchanger and Ca pump on the sarcolemma. The capacity for Ca exchange via the Ca channel increases during postnatal development as the channel density increases. In the neonatal heart, exchange via the Na/Ca exchange appears to be more substantial from the observations of the larger post-rest potentiation at long rest intervals (1- 100 s) (Figure 4.7B) and of the further increase in this post-rest potentiation by raising priming frequency (Figure 4.6A) and external Ca (Figure 4.6B) in 3 week old. This is consistent with evidence that Na/Ca exchanger is more active in the neonatal heart since the activity of Na/K ATPase which extrudes Na from the cell is greater in the newborn (Akera, 1972; Marsh, 1981; Nakanishi and Jarmakani, 1981; Park, 1981). Increase in stimulation frequency presumably mediates Ca loading by intracellular accumulation of Na (Cohen and Fozzard, 1982; Lee and Dagostino, 1982) which in turn reduces Ca efflux and enhances the influx of Ca by passive Na/Ca exchange (Reuter, 1974; Mullins, 1979; Johnson and Kootsey, 1985; Daut, 1982; Sheu and Fozzard, 1982; Chapman et al., 1983).

Complete absence of the T-tubular system appeared to account for the insensitivity of postnatal muscle to ryanodine, which was described first by Penefsky (1974a, b). However, this is unlikely to be the explanation for the insensitivity of the neonatal papillary muscles to ryanodine (Figure 4.7A) because atrial muscles, which also lack the T-tubular system initially and remain sparse during development, are sensitive to ryanodine (Chapter V).

A more likely explanation is that release of Ca via the embryonic form of the ryanodine receptor on the SR is not functionally blocked by ryanodine and that ryanodine sensitivity develops concurrently with incorporation of the adult form of the channel (Michalak, 1988; Pegg and Michalak, 1987). Since the T-tubular Ca channel may confer voltage sensitivity to the ryanodine receptors, it may very well be that the sensitivity of the ryanodine receptor to ryanodine blockade may develop concurrently with development of the T-tubules.

In conclusion, primitive intracellular stores exchange with external Ca via Ca channels and Na/Ca exchange and participate in E-C coupling at a stage of development when extracellular Ca is the major contribution to activator Ca. Progressively Ca storage increases and intracellular Ca becomes the major contributor to activator Ca. Then extracellular Ca exchange dynamically with internal stores to maintain the internal source of activator Ca for contraction.

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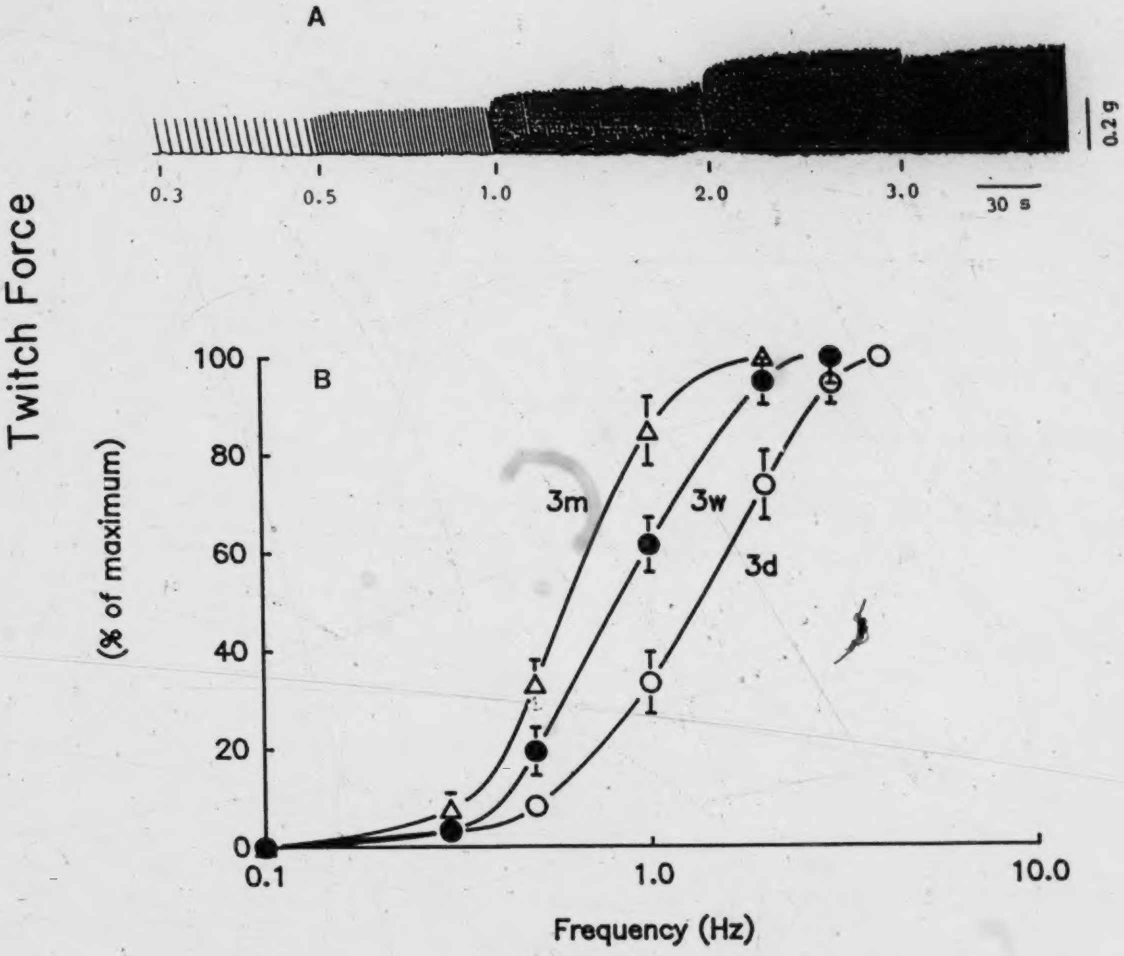

Figure 4.1. A), Isometric twitch contractions of adult papillary muscles, shown as a typical example, recorded at progressively higher rates of stimulation at 2.8 mM Ca, 37°C. B), Mean (±S.E.M.) of the steady state contractile force was expressed as a percentage of maximum force at the optimal frequency and plotted as a function of stimulation rate for 7 - 11 papillary muscles from 3 days (○), 3 weeks (●) and 3 months (△) old rabbits.

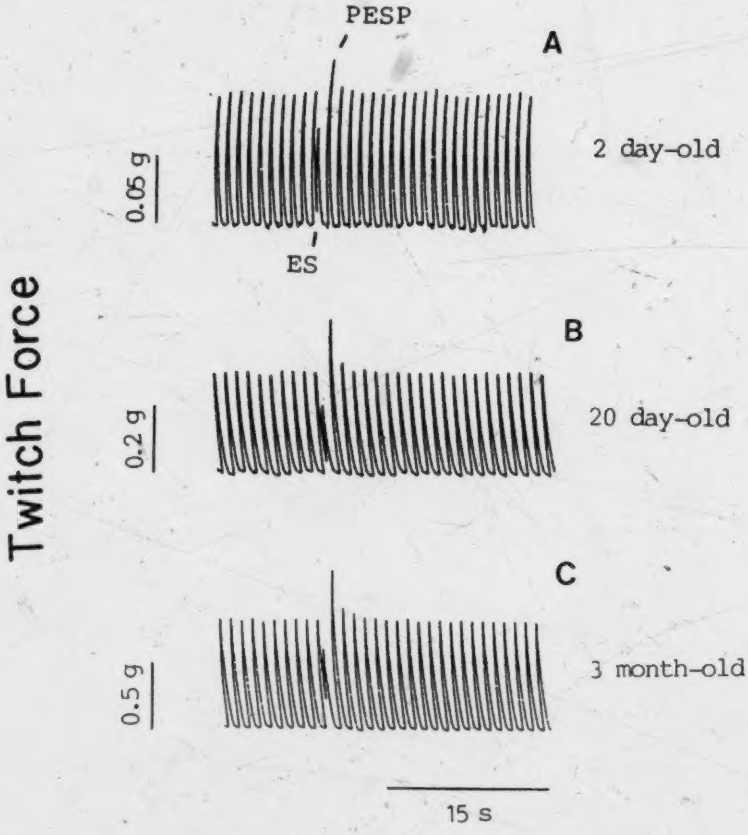

Figure 4.2. Comparison of extrasystolic force in papillary muscles from the newborn (A), juvenile (B) and adult (C) rabbits at 2.8 Ca. Interposing extrasystoles, i.e. very short intervals (< 1 s), on the stimulus train (1.0 Hz) caused a small force of extrasystolic twitch itself (ES) and potentiation of post-extrasystolic twitch force (PESP). ES is relatively greater in the newborn than the maturing hearts. PESP in the neonate was smaller than that in the older groups.

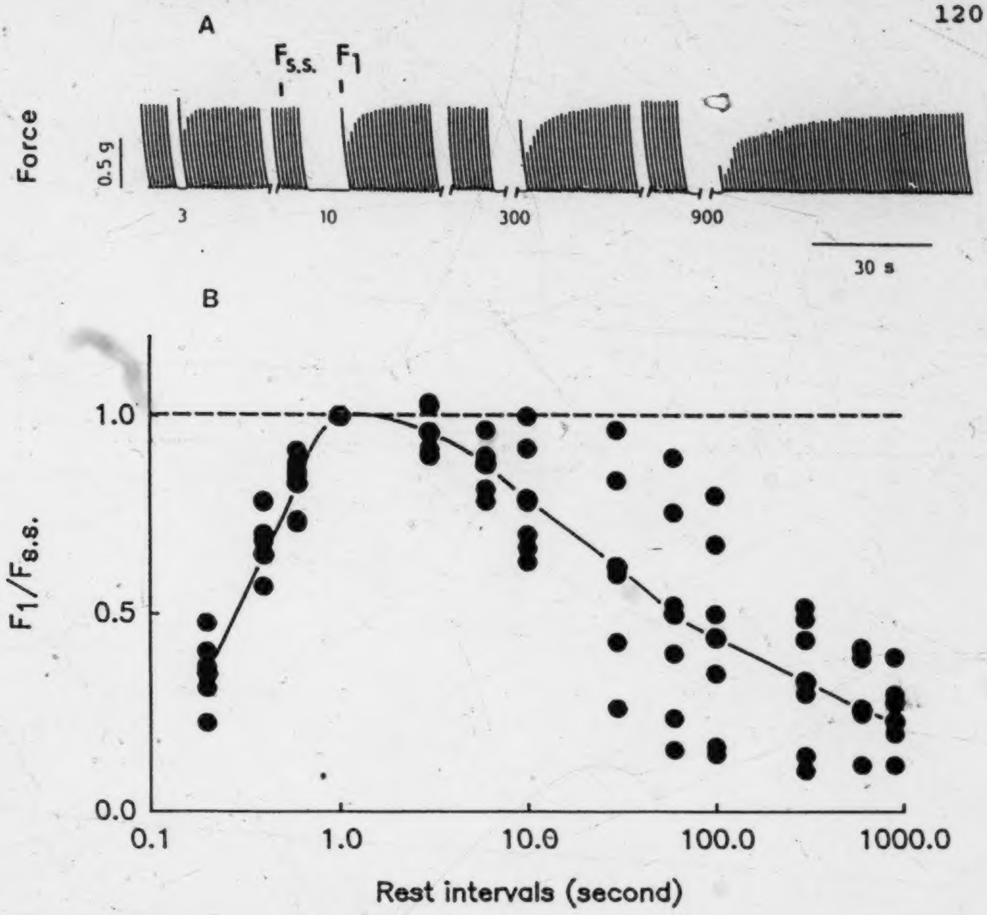

Figure 4.3. Mechanical restitution in adult papillary muscles. A), a typical example of influence of resting intervals on post-rest contractile force (F_1). The rest intervals varied from 3 to 900 s, as indicated below each force trace. $[Ca]_o$ was 2.8 mM and priming stimulation frequency was 1 Hz. B), F_1 from 7 individual experiments was expressed as a ratio of the steady state force prior to the rest interval and plotted as a function of the rest interval on a semi-logarithmic scale. The solid line connected the mean values at different rest intervals. The dashed line indicated steady state force level.

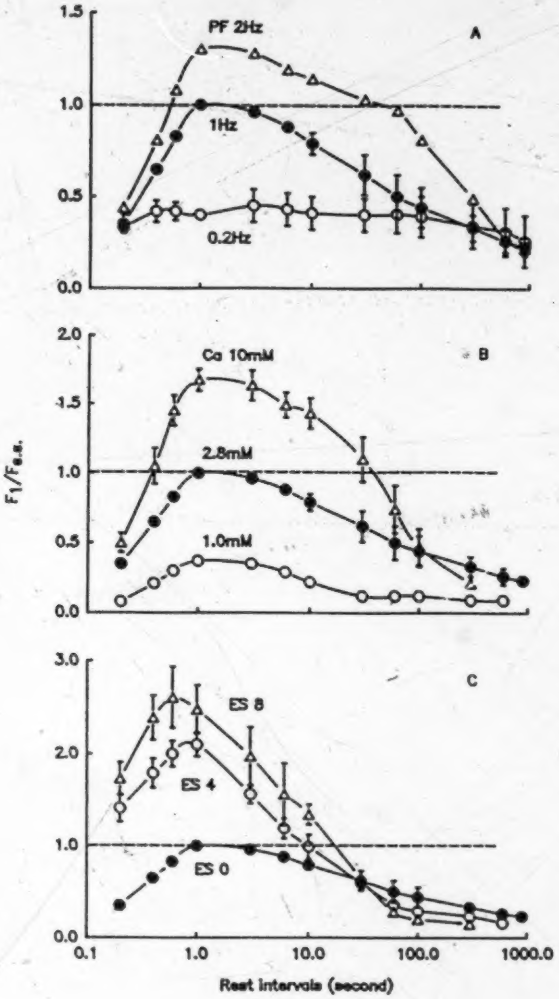

Figure 4.4. The influence of manipulation of exchange of intracellular and extracellular Ca compartments on mechanical restitution in adult papillary muscles by A) priming frequency, B) $[Ca]_o$ and C) 4 - 8 extrasystolic contractions. Note the different Y axis scales in A, B and C. Results are taken from 2 - 7 papillary muscles and shown as X ± S.E.M. PF: priming frequency. ES: extrasystole. The dashed line indicated steady state force level at $[Ca]_o$ 2.8 mM and priming frequency of 1.0 Hz.

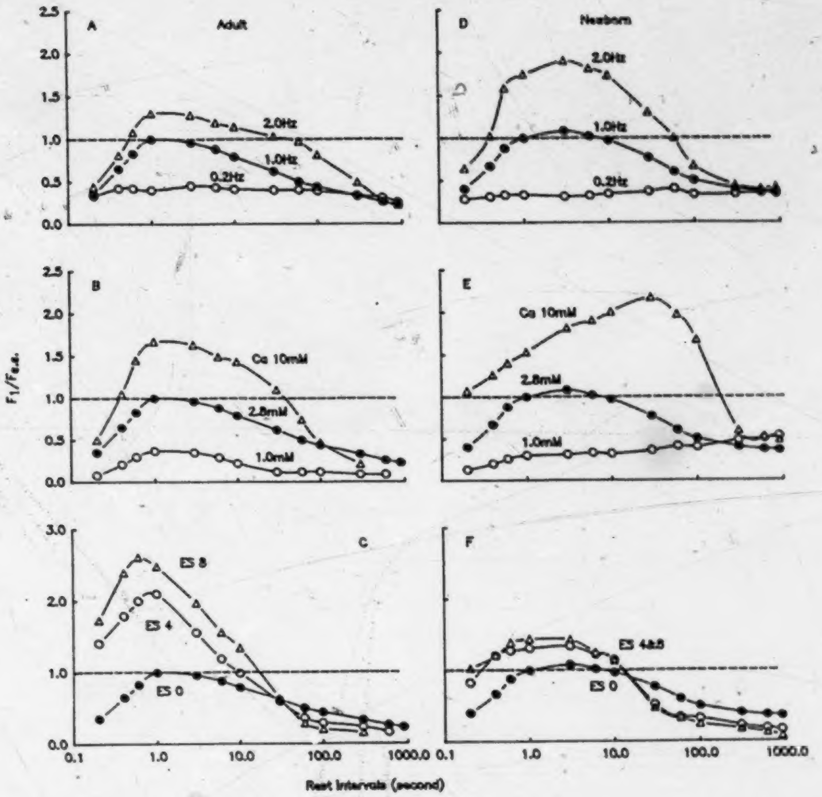

Figure 4.5. Comparison of the effects of priming frequency, $[Ca]_o$, and interposing extrasystolic contractions on mechanical restitution in papillary muscles from the adult (A, B and C, respectively) and newborn (D, E and F, respectively) rabbits. For comparison, the mean mechanical restitution curves from the adult muscles shown in Figure 4.4. were replotted with the same Y axis to that from the neonatal muscles under the same conditions of loading of intracellular Ca stores. Mean curves at 1.0 Hz priming frequency and 2.8 mM Ca from the adult (n = 7) and newborn (n = 5) muscles were represented by filled circles in all the graphs.

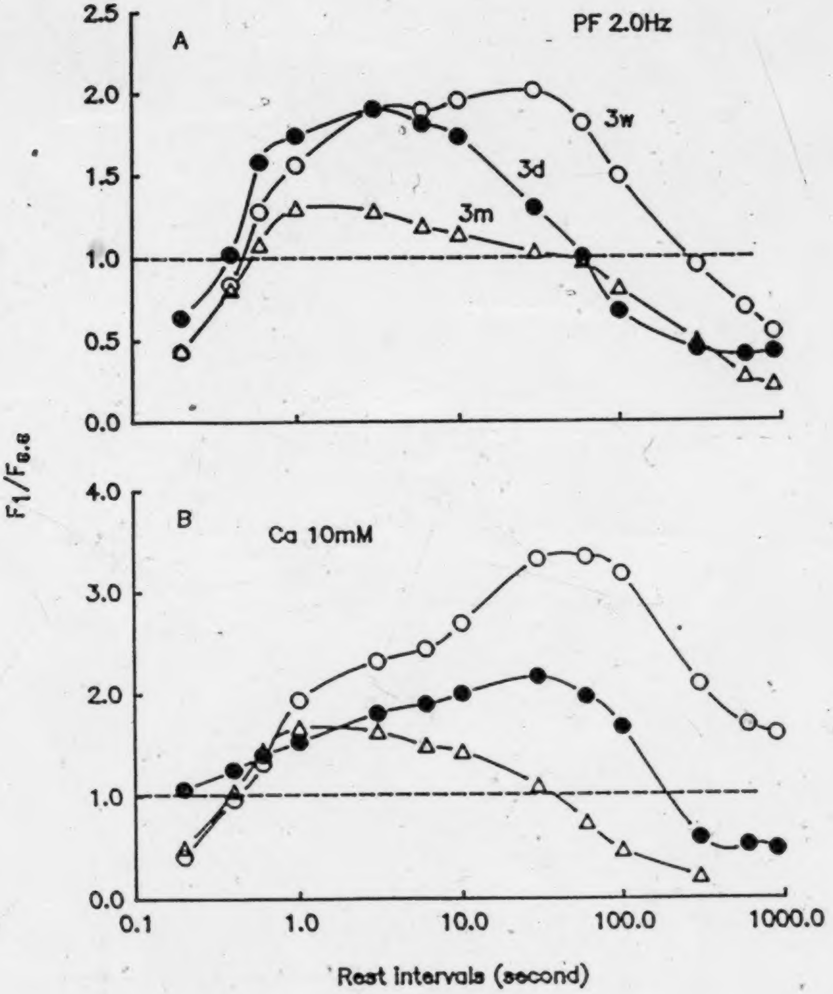

Figure 4.6. Comparison of mechanical restitution at A) high priming frequency (2.0 Hz) and B) high $[Ca]_o$ (10 mM) in papillary muscles from postnatal developing rabbits. Potentiation of post-rest contractile force at intervals between 10-100 s increased from 3 days (●) to 3 weeks (○) after birth, and then declined in the young adult (3 month-old, △).

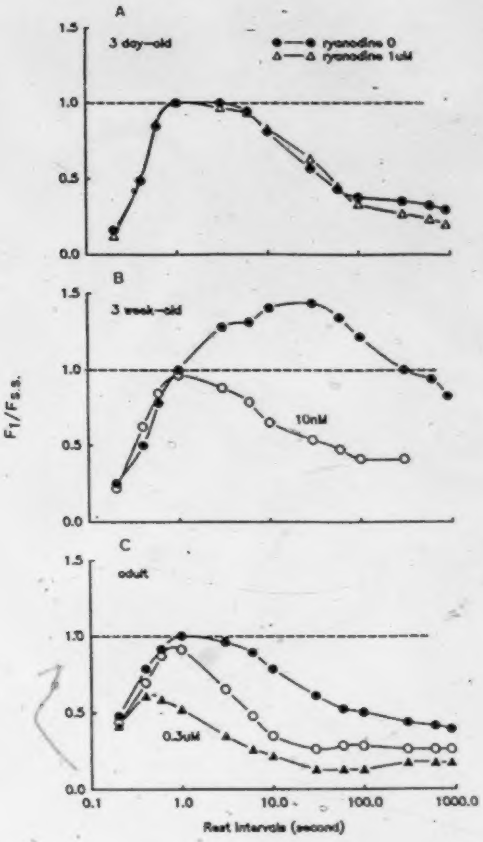

Figure 4.7. Comparison of effects of ryanodine on mechanical restitution in papillary muscles from A) 3 days, B) 3 weeks and C) 3 months old rabbits. Note that post-rest contractile force in the neonatal muscle (A) was insensitive to ryanodine even at very high concentration (1 uM). In contrast, a low concentration of ryanodine (10 uM) reduced post-rest contraction at intervals greater than 3 s without affecting steady state force in the older groups (B and C). Effects of ryanodine were concentration dependent in maturing muscles; an example was shown in C. The data shown were typical examples from individual experiments of each age group.

CHAPTER V. Ca COMPARTMENTALIZATION IN THE ATRIAL MUSCLES DURING POSTNATAL DEVELOPMENT*

Z.-P. Feng, T. Gordon and W.F. Dryden.

5.1. INTRODUCTION

In mammalian hearts, various Ca sources contribute to activation of contractile protein (Katz, 1970; Nayer and Merrilless, 1971; Kaufmann et al., 1974). External Ca entering cells via 1,4-dihydropyridine-sensitive Ca channels is implicated in the beat to beat control of contractile tension and also involved in a positive staircase phenomenon observed in most mammalian hearts (McCans et al., 1974; Bayer et al., 1975; Bennett and Seifen, 1979; Kennedy and Seifen, 1985). The latter is apparently due to Ca influx via Ca channels and the Na/Ca exchanger (Trautwein, 1973; Reuter 1974; Weidmann, 1974; Langer, 1978 and 1980; Temma et al., 1981). The intracellular Ca stores in the sarcoplasmic reticulum (SR) contribute activator Ca for muscle contraction and sequester Ca during relaxation. Ca is also extruded from the cell by the ATP-dependent Ca pump and Na/Ca exchanger in the sarcolemma

* The results of this chapter have been published in abstract form [Feng Z-P, Gordon T and Dryden WF. Excitation-contraction (E-C) coupling in postnatal cardiac muscle in the rabbit. FASEB J. 1989, 3(4):A986.].

(Reviewed by Dhalla et al., 1982).

In the adult rabbit atria, internal stores of Ca are major source of activator Ca for contraction while in the ventricular muscles, extracellular of Ca contributes relatively more (Siegl and McNeill, 1982; Bers, 1985; Boucek et al., 1987). Nevertheless, studies of post-rest contractions in atria indicate that exchange between extracellular and intracellular Ca loads the intracellular stores which can be seen as a potentiation of post-rest contractions (Manring and Hollander, 1971; Frank and Sleaton, 1975; Temma et al., 1981; Hilgemann et al., 1983; Bers 1985). The post-rest potentiation of contraction reflects a larger amount of Ca available for release from the intracellular stores upon resumption of stimulation (Tillisch et al., 1979; Temma et al., 1981; Hilgemann et al., 1983; Sutko et al., 1986).

There are also structural differences of the SR and T-tubular system between the atrial and ventricular muscles. In atria, the T-tubular system is sparse and the SR is always junctionally associated only with the external sarcolemmal membrane (Ishikawa and Yamada, 1976). In contrast, association between SR and T-tubular membranes in ventricular muscles develops coincidentally with a decrease in association of the SR with the sarcolemma during postnatal development (Ishikawa and Yamada, 1976; Page and Buecker, 1981; Maylie, 1982). The junctional faces of the SR associated with either the sarcolemma or the T-tubule are the sites of Ca release from the SR (Maylie, 1982), but the Ca release mechanism of them may differ since that relative contribution of intracellular Ca to extracellular Ca is different in the atrial from that in the ventricles (Fabiato and Fabiato, 1978; Bers et al., 1981; Fabiato, 1982).

This study was undertaken to elucidate the development of E-C coupling and relative contributions of internal and external Ca to contraction in atria, and to determine whether internal Ca is already a major determinant of contractile force in the immature atrial muscle in contrast to ventricles. We have examined the influence of external Ca on steady state and post-rest contractions by manipulating Ca influx with Ca concentration, stimulation and drugs that influence the voltage-sensitive Ca channels on the sarcolemma and Ca release channels on the SR.

5.2. RESULTS

5.2.1. Steady state contraction

5.2.1.1. Force-frequency relationship

Contractile force increased with the rate of stimulation frequency from 0.1 Hz to 3 Hz at which a maximal force was developed in immature and mature atrial muscles of the growing rabbit. The positive staircase effect of stimulation frequency is illustrated in the example taken from a 3 month rabbit (Figure 5.1A) which was very similar in immature muscles as shown by force-frequency curves in Figure 5.1B.

(Figure 5.1 near here)

One Hz of stimulation frequency, at which approximate 50% of the maximal contractile force was developed in each age group, was selected as the stimulation rate for steady state contraction in the subsequent studies (see also Chapter IV).

5.2.1.2. Inotropic effects of $[ca]_o$ and dihydropyridines

The inotropic effects of $[Ca]_o$ and dihydropyridines (Figure 5.2, 5.3 and 5.4) are age-dependent in contrast to the effects of stimulation frequency (Figure 5.1). The positive inotropic effects of $[Ca_o$ and Bay K8644 increased in parallel with age. The effectiveness of each increased by a factor of 2 with the effect of high Ca increasing the maximum steady state force by 1000% at 3 weeks of age and to 2000% in the young adult (Figure 5.2A), and Bay K8644, 100 to 200% at 3 weeks and 3 months of age (Figure 5.3A). Sensitivities of the atrial muscles to $[Ca]_o$ and Bay K8644 did not change during development as shown in Figure 5.2B and 5.3B, respectively.

(Figure 5.2 and 5.3 near here)

The increases in positive inotropic effects of $[Ca]_o$ and Bay K8644 are consistent witn an increase in numbers of Ca channels in the sarcolemma during postnatal development as suggested by an increase in Ca current recorded in atrial cells (Yabet et al., 1987) and found experimentally in ventricular homogenates (Feng et al., 1988 and 1989). Nevertheless, relative contribution of extracellular Ca to contraction decreased with age since steady state force was less affected by nifedipine in mature muscles (Figure 5.4).

(Figure 5.4 near here)

5.2.2. Post-rest contraction

Post-rest contractile force (F_1) after each rest interval (0.2 - 900 s) interposed on steady state force ($F_{s.s.}$), at a constant stimulation rate of 1 Hz, was traced on paced atrial muscles from the newborn, juvenile and adult hearts. In the adult muscle, contractile tension depends on the interval between beats as a result of the dynamic distribution of Ca between intracellular and extracellular Ca pools. Post-rest contractions were potentiated relative to steady state force ($F_{s.s.}$) at rest intervals of 3 - 100 seconds (Figure 5.5B) as a result of accumulation of intracellular Ca. Similarly, in the neonatal muscle (Figure 5.5A), post-rest contractions are potentiated by interruptions of rest intervals showing that intracellular Ca stores in the immature muscles are able to accumulate and release Ca. However, the recovery phase following each F_1 on beat to beat base in the newborn heart was much slower as compared to that in the adult. This difference indicates that accumulation of Ca in intracellular stores is initially limited in the immature atrial muscle.

(Figure 5.5 near here)

The data from 7 experiments in the young adult group are collated and plotted in Figure 5.6A. Mean values are shown as solid line at a priming frequency of 1 Hz and 2.8 mM Ca where three phases may be discerned with respect to duration of rest intervals: 1) a phase between 0.2 and 10 seconds where post-rest force increases to a plateau, 2) a second phase of potentiation at intervals between 10 - 100 seconds where a second peak force was obtained at interval of 100 s, and 3) a final phase where post-rest force declines, as described previously by Schouten et al. (1987) in rat ventricular muscles and Feng et al. (1989) in rabbit muscles. According to a three compartment model of Schouten et al. (1987), these phases may be accounted for by 1) movement of intracellular Ca from uptake to release compartments, 2) replenishment of Ca stores by external Ca from Na/Ca exchange, and 3) loss of Ca from internal to external compartments via Na/Ca exchange and to a lesser extent by the sarcolemmal Ca ATPase.

In the adult, increase in the priming frequency potentiates the post-rest contraction at each rest interval, consistent with an increase in sarcolemmal Ca influx via Ca channels and the Na/Ca exchanger (Figure 5.6B). The post-rest contraction in the second phase was enhanced to reach the maximum at 10 s and force did not decline even after long rest intervals (300 - 900 s). The constant post-rest contractile force at high frequency at intervals from 10 - 900 s could be a result of saturation of one of the steps in Ca transport or of the contractile filaments. The influence of raising $[Ca]_o$ on the post-rest contractions (Figure 5.6C) was qualitatively similar to that of the priming frequency but the potentiating effect of $[Ca]_o$ was almost twice as large as the priming frequency.

(Figure 5.6 near here)

Interpolation of extrasystoles before each rest interval produced a very pronounced potentiation of post-rest contractions at all intervals but most particularly at short rest intervals (0.2 - 10 s) (Figure 5.6D). This post-extrasystolic potentiation can be accounted for by Ca influx via the Ca channels, which are opened during extrasystoles, to load the internal Ca stores and to be available for release.

The obvious difference in the newborn muscles from adult is the smaller post-rest potentiation, particularly at long intervals (Figure 5.6E-H). In addition, manipulation of Ca influx via Ca channels and Na/Ca exchanger is far less effective in increasing post-rest potentiation (Figure 5.6E-H). Note, however, that external Ca was more effective in producing potentiation and that post-rest potentiation was ever achieved at long intervals (Figure 5.6G).

In summary, as animal matures, the characteristic two phases of potentiation of contractile force with maximum at 10 and 100 s rest intervals appears (Figure 5.7A). The post-rest potentiation at intervals of 10 - 100 s may be accounted for by increasing activity of Na/Ca exchanger in the adult since it is increased by high priming frequency,

$[Ca]_o$ and extrasystoles (Figure 5.7B-D). Another interesting property of this emergence is that internal Ca is not readily exchanged with external Ca at long intervals where post-rest contractions are still potentiated in the adult (Figure 5.7B, C). If Ca loading is promoted by extrasystoles, on the other hand, the declined potentiation at long intervals (100 - 900 s) provides further evidence to suggest that Na/Ca exchanger is an important factor in reducing the internal stores during long rest intervals (Figure 5.7D). Thus these results were consistent with increases in Ca channel density (see also Figure 5.2), Na/Ca exchange activity and internal storage capacity.

(Figure 5.7 near here)

5.2.3. Effects of ryanodine

Ryanodine is known as a specific blocker for Ca release from the SR by acting on the ryanodine sensitive Ca channel on the SR and therefore reduces the contractile tension. Post-rest potentiation developed after intervals (> 3 s) was reduced by a low concentration of ryanodine (10 nM) in the muscles from all three age groups (Figure 5.8). These data indicate that the post-rest potentiation is due to Ca released from the SR and that the intracellular ryanodine sensitive Ca channels are present in atrial muscles at birth.

(Figure 5.8 near here)

5.3. DISCUSSION

The present studies indicated that, in atrial muscles of rabbits 1) the DHP-sensitive Ca channels are functionally mature in the neonatal muscles but the full adult complement of the channels is not yet attained, 2) the direct reliance of the contraction on the external Ca is relatively greater in the neonate and decreases with age, 3) intracellular Ca stores also contribute to activator Ca for contraction in the newborn muscles and the capacity of extracellular Ca to load these stores increases progressively during postnatal development with increase in Ca channel density and the activity of Na/Ca exchanger, and 4) Ca release from internal stores at all ages is ryanodine sensitive even though the T-tubular system is sparse.

5.3.1. Steady state force

Consistent with our previous findings (Feng et al., 1988; Chapter III) that the population of the DHP-sensitive Ca channels in ventricular muscles increased during early postnatal development of rabbits, there is the 2-fold increase in the effectiveness of Bay K8644 and $[Ca]_0$ on steady state contraction with unchanged sensitivities. These changes may also reflect an increase in the Ca channel density, although the Bay K8644-binding sites on the Ca channel may mature earlier in the atria than the ventricular muscles. The decreased effectiveness of nifedipine in reducing steady state force in maturing muscles indicates a decrease in the reliance of the mature hearts to $[Ca]_o$ despite the increase in the Ca channel population.

5.3.2. Mechanical restitution

Mechanical restitution curve in adult atrial muscles of rabbits is composed cf two phases of increasing post-rest potentiation at intervals of 0.2 - 10 s and 10 - 100 s, respectively, which differs from that in ventricular muscles (Feng et al., 1989; Chapter IV). In the latter, there is no post-rest potentiation of force at any resting interval. This cell-type dependent restitution indicates that Ca transport during E-C coupling is fundamentally different which may be accounted for by a higher density of the SR and sparse T-tubular membranes in the atrial myocytes (Ishikawa and Yamada, 1976).

As described in the result section, the first increase phase of post-rest contraction occurring during intervals of 0.2 to 10 s is strongly increased by interpolation of extrasystoles before each interval. The post-extrasystolic potentiation in the adult atrial muscle agrees well with that found in other species by Hoffman et al. (1956, dog), Garb and Penna (1956, cat), and Schouten et al. (1987, rat). The potentiation is presumed to be the as a result of an accumulation of the inflow Ca via Ca channels into the intracellular stores and then transport from the uptake to release compartments (Manring and Hollander, 1971; Kaufmann et al., 1974; Edman et al., 1976; Wohlfart, 1979; Schouten et al., 1987). The second increase phase to the peak at about 100 s rest interval is potentiated by increases of priming stimulation frequency, which induces an intracellular Na accumulation, and $[Ca]_o$. The combination effects of the Na/Ca exchange mechanism and the Na/K pump to allow a small diastolic influx of Ca, have been postulated to accounted for this peak post-rest contraction at interval of 100 s (Mullins, 1979; Sheu and Fozzard, 1982; Schouten et al., 1987). Finally, the third decline phase occurring at intervals longer than 100 s reflects a leak of Ca from internal stores via sarcolemma. The post-rest potentiation at intervals of 3 - 900 s was diminished by a low concentration of ryanodine showing the SR is a major source of activator Ca for contraction.

In the neonatal atria, the finding that post-rest contraction at intervals of 10 - 100 s was potentiated shows that internal Ca contributes activator Ca for contraction at birth and is consistent to the observations by Boucek et al. (1987). In addition, the post-rest potentiation is diminished by ryanodine (Figure 5.8A). This provide further evidence that intracellular Ca store is present and functions as a source of activator Ca at the time of birth. In contrast, the ryanodine induced blockade of Ca release seen as the decrease in post-rest contractile force in the neonatal atrial muscles indicates that the ryanodine-sensitive Ca channel matures earlier in the atrial than papillary muscles. In the latter, post-rest contraction are insensitive to ryanodine (Chapter IV).

The amplitude of the post-rest potentiation at intervals of 10 - 100 s increased significantly in the adult atria. Moreover, effects of high $[Ca]_o$ and priming frequency, which lead to a change in cellular Na/Ca ratio and facilitate the inward Ca transport via Na/Ca exchange (Li and Vassalle, 1983; Sutko et al., 1986), on the post-rest potentiation were also increased postnatally (Figure 5.6). Since immature atria require a higher $[Ca]_i$ in order to maximize SR Ca loading and release (Vornanen, 1985; Boucek et al., 1987), the small force potentiation in the neonatal muscle could be due to an insufficient Ca loading in the SR by Ca influx via the Na/Ca exchange. Studies on steady state contraction of the cardiac septal muscles in rabbits demonstrated that the inotropic responses of the neonatal muscles to varied $[Na]_o$ were greater than that in the adult (Nakanishi and Jarmakani, 1981; Uemura et al., 1985), although the net maximal Na/Ca exchange and Ca efflux rates of the sarcolemmal vesicles at the physiological condition was unchanged during postnatal development (Meno et al., 1988). These results suggested that Na/Ca exchange was more active in regulating ion transport in the immature that in the adult. Therefore, the increase in post-rest potentiation from the newborn to adult could reflect a corresponding increase in the capacity of intracellular Ca

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stores. In other words, the internal Ca stores are limited in their capacity in the neonatal atrial muscle and therefore Na/Ca exchange mechanism is predominant for Ca extrusion during rest interval since Ca loading in the SR is relatively small. Thereby, the post-rest potentiation in the immature atrial muscles is relatively less than in the adult.

In summary, intracellular Ca stores are relatively mature and contribute activator Ca for contraction in the neonatal atrial muscles. The ryanodine receptors on the SR are also functionally mature at the time of birth. During postnatal development, a decrease in the reliance of the external Ca for contraction occurs concurrently with an increase in the capacity of the internal Ca stores.

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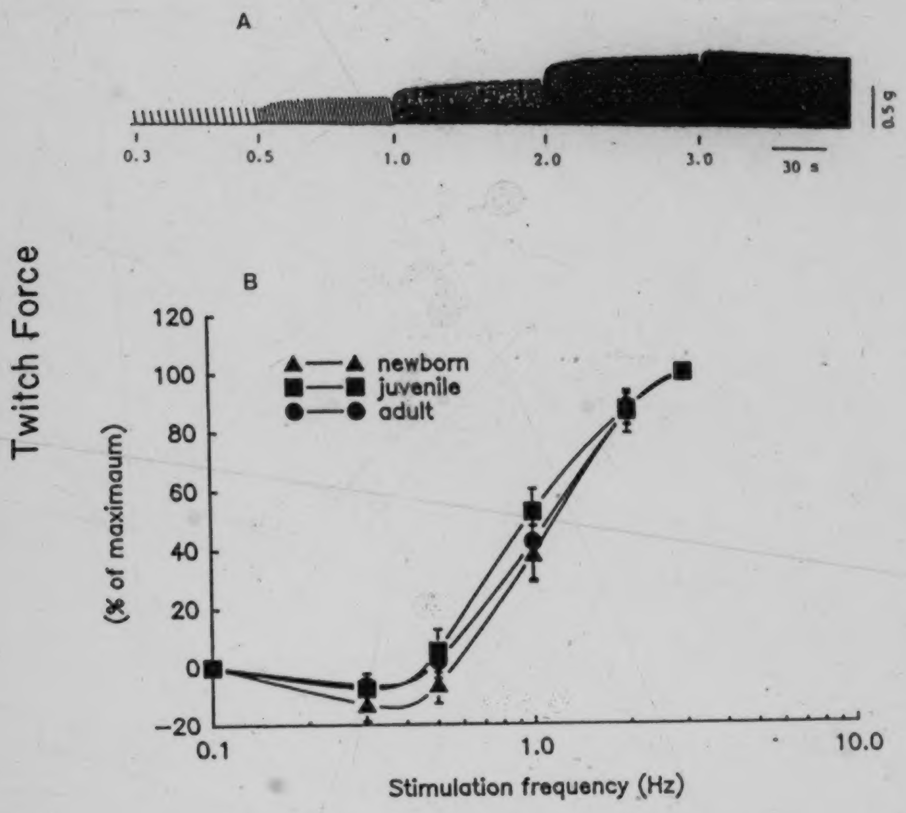

Figure 5.1. Force-frequency relationship of atrial muscles from the different age groups of the rabbit. A), Isometric twitch contractions of adult atria, shown as a typical example, recorded at progressively increased stimulation frequency from 0.3 to 3 Hz at 37°C, Ca 2.8 mM. B), Mean (±S.E.M.) of the steady state force was expressed as a percentage of maximum force at the optimal frequency and plotted as a function of stimulation frequency for 6 - 9 atrial muscles from newborn (▲), juvenile (■) and young adult (●) rabbits.

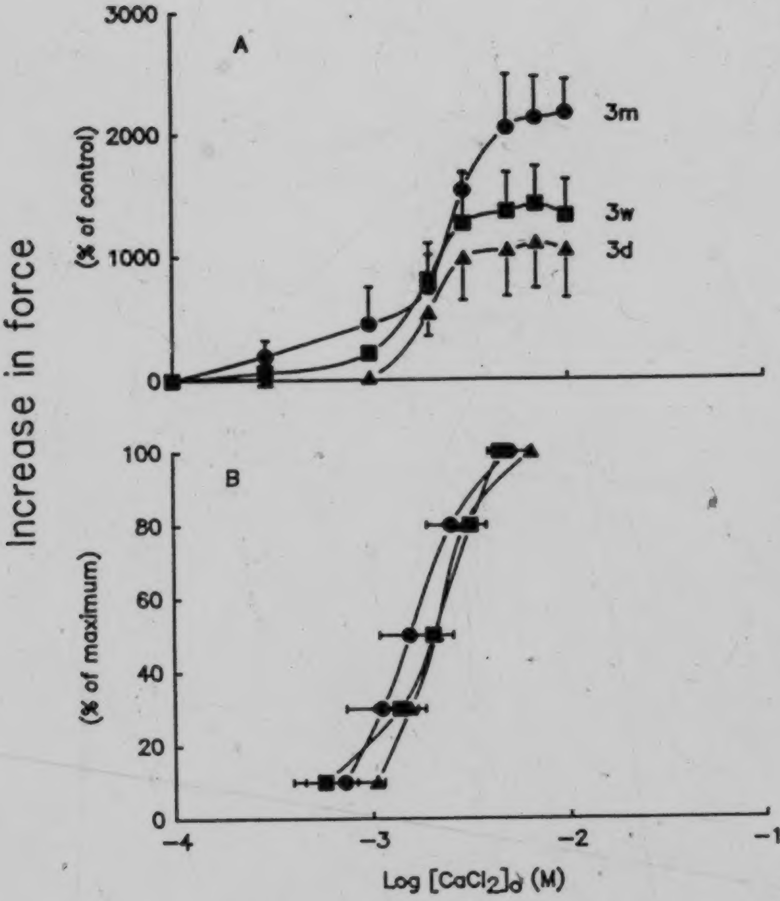

Figure 5.2. The positive inotropic effects of $[Ca]_o$ on atrial muscles from different ages of rabbits. A), the maximum effects of extracellular Ca was expressed as the percentage increase in steady state force at 1.0 Hz stimulation frequency above the control force obtained at 0.1 mM of $[Ca]_o$, and plotted as arithmetic mean (± S.E.M.) for 5 - 8 atrial muscles as a function of $[Ca]_o$. The maximal effects of $[Ca]_o$ decreased postnatally. B), The same data were replotted as mean concentration (± S.E.M.) for given response. There was no difference in each age group.

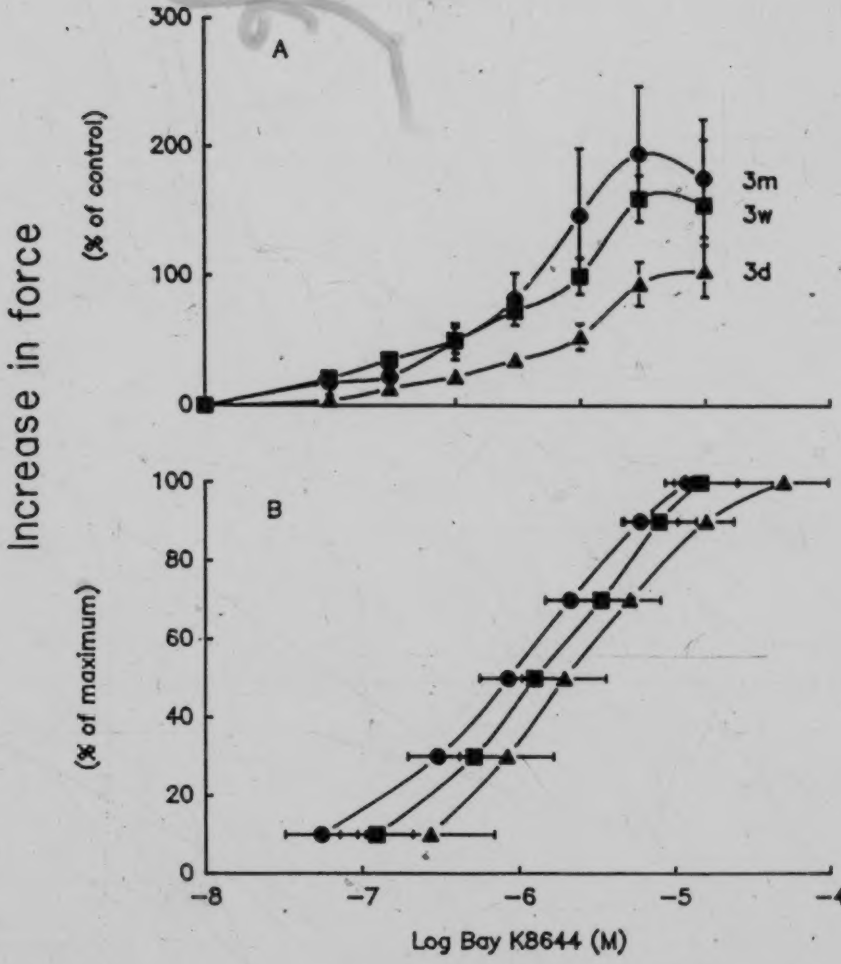

Figure 5.3. The positive inotropic effects of Bay K8644 on atrial muscles from different age groups. A), The arithmetic mean (± S.E.M.) of maximal percentage increase in steady state force in the presence of Bay K8644 was expressed as a percentage of the control force recorded in the absence of the drug, and found to increase progressively with age. B), The same data were replotted as mean concentration (±S.E.M.) for given response. There was no difference in each age group.

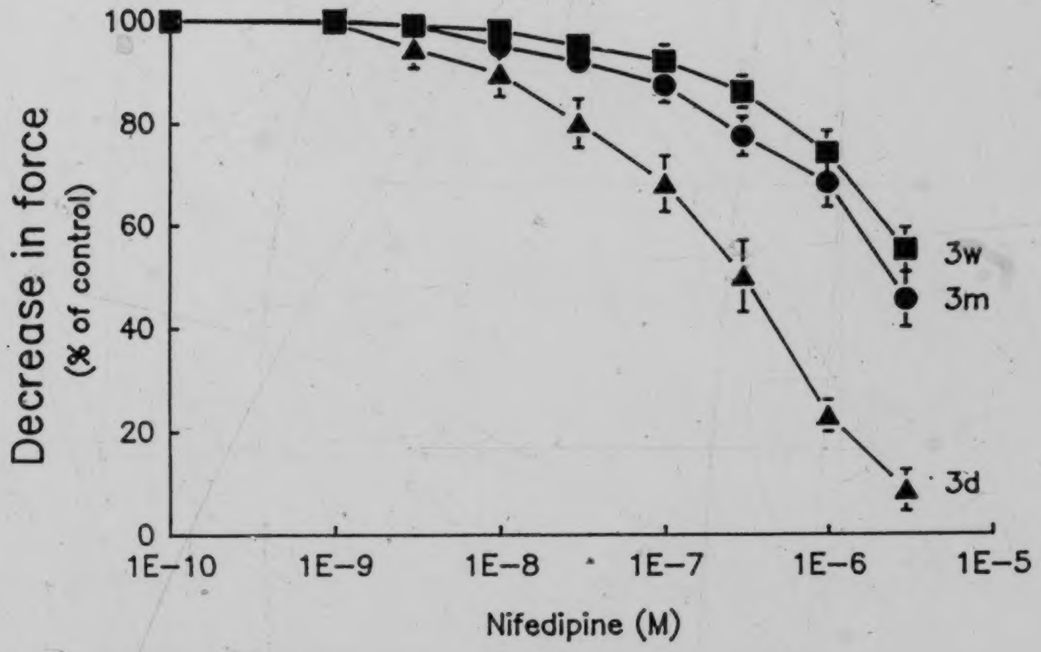

Figure 5.4. Concentration-effect curves of the negative inotropic response of developing atrial muscles to nifedipine. The data were plotted as the mean (± S.E.M.) percentage decrease in steady state force of the control force recorded before nifedipine was added. The nifedipine induced inhibition on the twitch force at each concentration of the drug in the newborn was greater than that in the older groups.

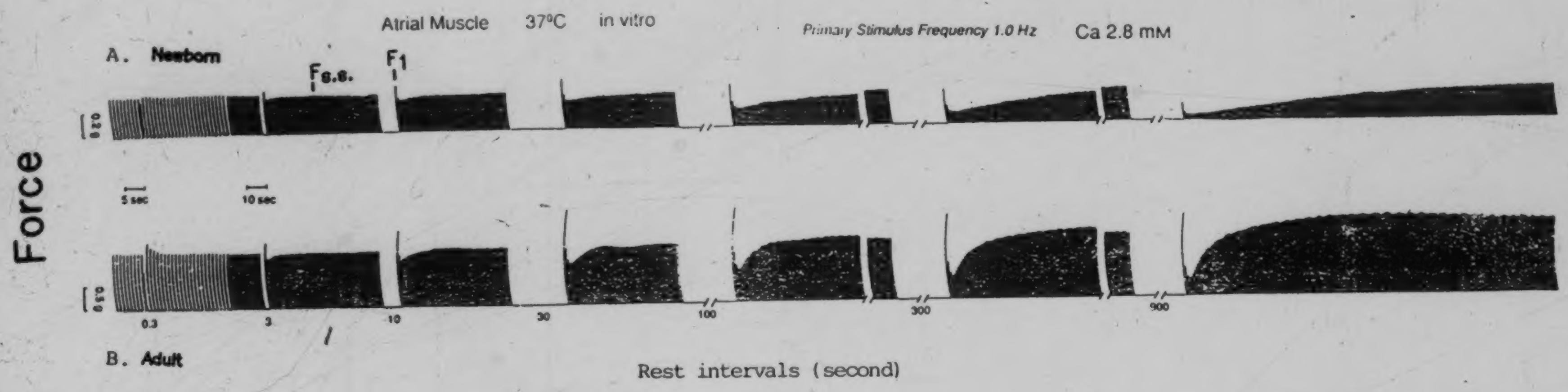

Figure 5.5. Post-rest contractions of (A) the neonatal and (B) adult atrial muscles in response to progressively increase in time of rest intervals, at 1.0 Hz priming stimulation frequency, 2.8 mM Ca and 37°C. F_1: post-rest contractile force. $F_{s.s.}$: steady state force.

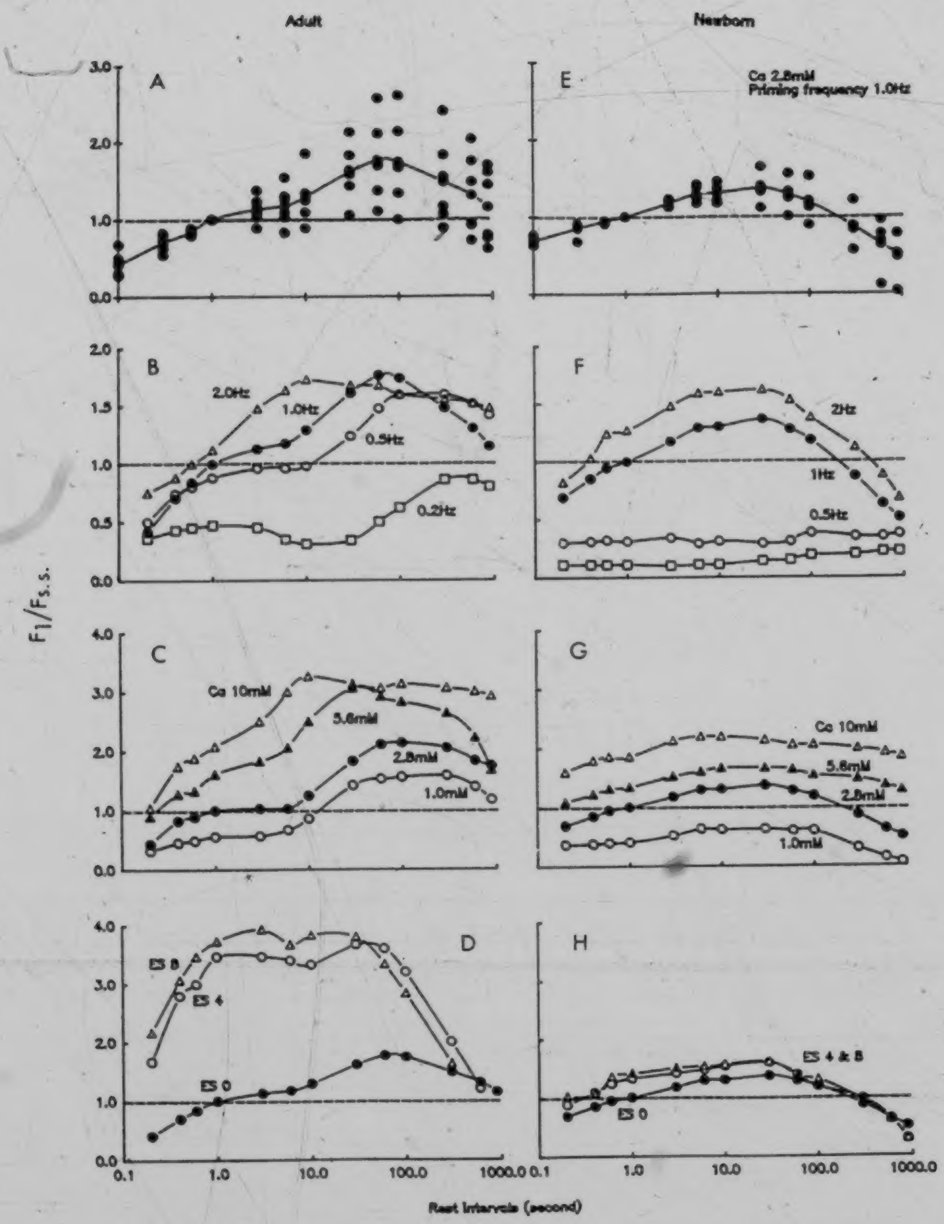

Figure 5.6. Mechanical restitution and effects of priming frequency, $[Ca]_o$ and interposing extrasystolic contractions on the mechanical restitution in atrial muscles from the adult (A, B, C and D, respectively) and newborn (E, F, G and H,

respectively) rabbits. For comparison, the mechanical restitution curves obtained under the same conditions of loading of intracellular Ca stores in the adult and newborn were plotted with the same Y axis. A) and E), Post-rest contractile force (F_1) of atrial muscles from the adult (A, n = 6) and newborn (F, n = 4), at 1.0 Hz priming stimulation frequency and 2.8 mM Ca, was expressed as a ratio of the steady state force prior to the rest intervals and plotted as a function of the rest interval on a semi-logarithmic scale. The solid line connected the mean values at different rest intervals. The dashed line indicated steady state force level. Mechanical restitution curves measured in (B and F) varied priming frequency (at 2.8 mM Ca), (C and G) $[Ca]_o$ (at 1.0 Hz priming frequency), and (D and H) 4 - 8 extrasystolic contractions (at 2.8 mM Ca and 1.0 Hz priming frequency).

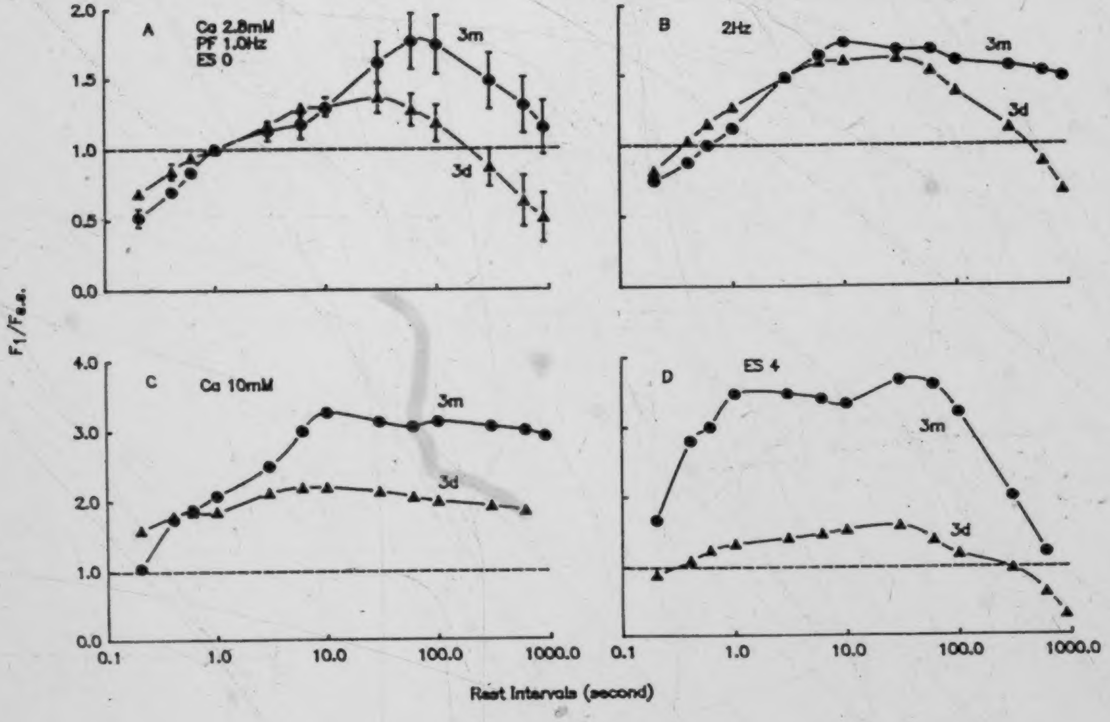

Figure 5.7. Comparison of mechanical restitution in atrial muscles from the adult and newborn rabbits. For comparison, A), the mean mechanical restitution curves from Figure 5.6A and E, were replotted as X ± S.E.M. Effects of B) high priming frequency (2.0 Hz), C) high $[Ca]_o$ (10 mM) and D) 4 interposed extrasystolic contractions on post-rest contractions of the neonatal muscles were limited as compared to that in the adults.

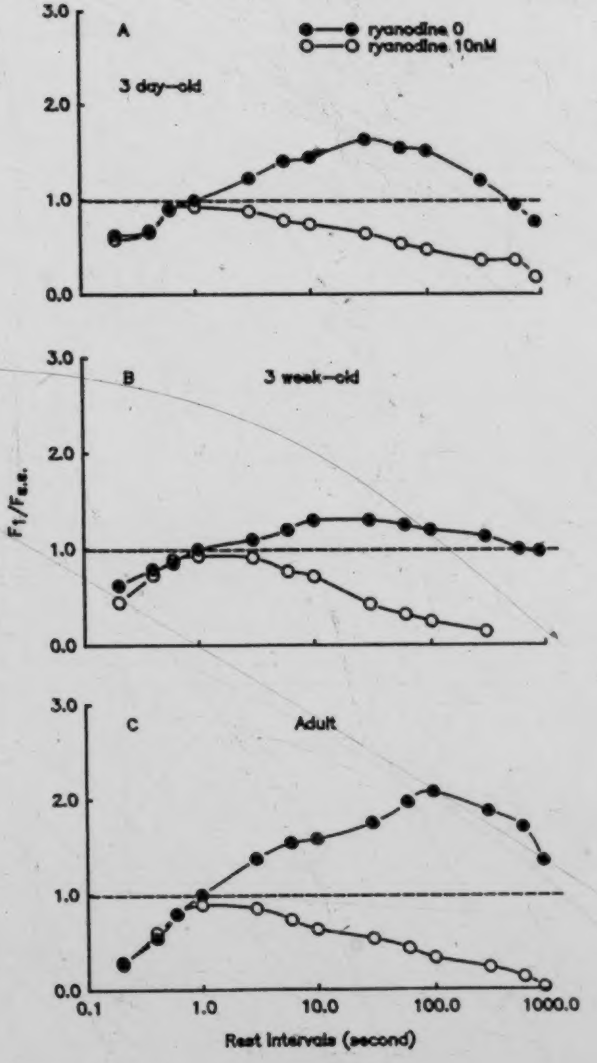

Figure 5.8. Comparison of effects of ryanodine on mechanical restitution in atrial muscles from A) 3 days, B) 3 weeks and C) 3 months old rabbits. Post-rest potentiation of contraction was diminished by a low concentration of ryanodine (10 nM) in all age groups. The data shown were typical examples from individual experiments of each age group.

CHAPTER VI. POSTNATAL DEVELOPMENT OF ADRENERGIC RESPONSIVENESS IN VENTRICULAR MUSCLES*

Z.-P. Feng, W.F. Dryden and T. Gordon

6.1. INTRODUCTION

Several factors may contribute to age-related changes in the beta-adrenoceptor mediated inotropic response of the postnatal developing heart (Chen et al., 1979; Vulliemoz et al., 1984). These include regulation of numbers of adrenoceptors and changes in the affinity of these receptors to catecholamines, as well as the efficiency of transduction from receptor binding to the ultimate effect, namely the contraction of myofibrils.

Studies of the functional response to beta-adrenoceptor agonists during development in a number of species, including chick (Higgins and Pappano, 1981), dog (Rockson et al., 1981; Spinelli et al., 1986), mouse (Chen et al., 1979; Roeske and Wildenthal, 1981), rabbit (Park et al., 1980; Nishioka et al., 1981) and rat (Standen, 1978 and Amerini et al., 1985), although sometimes contradictory, led to the tentative conclusion that the numbers of receptors increased with postnatal age. Alexander et al. (1982) correlated this increase with the time of development of the sympathetic has been accepted innervation of the heart (reviewed by Pappano, 1977). Consistent with this, numbers of tritiated dihydroalprenolol ([^{3}H]DHA) binding sites increased in the rat (Baker and Potter, 1980). Yet, data from radioligand binding studies are not always consistent. For example, numbers of cardiac beta-adrenoceptors measured in the adult dog were less than those in the 2 week old neonate, even though functional studies suggested that they were greater (Rockson et al, 1981). A more detailed description of the time course of developmental changes in beta-adrenoceptors in another species, mouse, suggests that this discrepancy may be explained by an observed increase in the site density which is followed by a decline to adult levels (Chen et al., 1979; Roeske and Wildenthal, 1981).

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In addition to the controversy regarding the beta-adrenoceptor density in the rabbit heart, there is little agreement regarding in the receptor affinity in postnatal development. Many studies conclude that the affinity remains unchanged (Chen et al., 1979; Baker and Potter, 1980; Nishioka et al., 1981; Rockson et al., 1981; Alexander et al., 1982; Whitsett et al., 1982; Fan and Banerjee, 1985). Yet some authors have reported a significant postnatal increase in the affinity of binding sites for [^{3}H]DHA on rat ventricular membranes (Bhalla et al., 1980), and others have noted an increase in the sensitivity of developing ventricular tissues to isoproterenol in several species (Au et al., 1980; Park et al., 1980; Higgins and Pappano, 1981).

The simplest approach to assess the relative contribution of numbers and affinity of beta-adrenoceptors is to carry out studies which attempt to correlate the inotropic response of developing cardiac muscles with the radioligand binding characteristics of the same tissues. This is the approach adopted in this study of the ventricular muscle in the neonatal rabbit heart. The results of this study have been communicated in abstract form (Feng et al., 1987).

6.2. MATERIALS AND METHODS

Correlative studies of the contractile response of left ventricular papillary muscles to the beta-adrenoceptor agonist, isoproterenol, and equilibrium binding assays of [^{3}H]DHA to homogenate preparations of ventricles and isolated ventricular myocytes were carried out using Dutch rabbits of either sex at 1-3 days, 20-23 days or 80-100 days of postnatal age.

6.2.1. Inotropic effects of isoproterenol

6.2.1.1. Experimental protocol

Rabbits were sacrificed by cervical dislocation, and each heart was rapidly removed and placed in Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10; pH 7.4) which was kept on ice and gassed with 95% O_2 and 5% CO_2. Ventricular papillary muscles were dissected free and mounted in 4 ml tissue baths containing oxygenated Krebs-Henseleit solution maintained at 37°C. Each papillary muscle was attached to a Grass isometric force-displacement transducer (FTO3B), coupled to a Grass polygraph (model 5) for recording, and stimulated via bipolar punctate platinum electrodes with 5 msec square-wave pulses of 2x threshold voltage at a frequency of 1 Hz. Muscle length was initially varied to establish the length-tension relationship. Length was then adjusted to provide twitch tension equal to 70% of maximum. A period of at least 1 hour was allowed to elapse to ensure stable performance of the tissues. Concentration-effect relationships for the beta-adrenoceptor agonist, isoproterenol, were obtained from cumulative addition of the drug to the tissue baths until maximal responses were obtained. The response to each concentration was allowed to plateau over a 5 minute interval before further addition of the drug to the bath.

6.2.1.2. Data analysis and statistics

The contractile responses to each dose were averaged for 8-13 preparations. The increase in tension was expressed as percentage of the initial tension and the arithmetic mean (±S.E.M.) calculated. The geometric mean, i.e. the mean of the log values ± standard error of the mean (EC_{50}), was calculated for the isoproterenol concentration required to elicit responses of 10, 30, 50 80 and 100% of the maximum responses as described in detail by Carpenter (1986).

Differences between mean values of maximal responses (T_{max}) and EC_{50} for each age group were examined using Duncan's multiple range test for unequal sample sizes and the differences were taken to be significant where $p < 0.05$ (Walpole, 1974; Dowdy and Wearden, 1983).

6.2.2. [^{3}H]DHA equilibrium binding

6.2.2.1. Preparations

Whole ventricular homogenates were chosen for the equilibrium binding study in preference to purified membrane preparations (Bambrick et al., 1988; Chapter II) as the protein/phospholipid ratio of membranes is age-dependent (Cohen and Zubenko, 1985) and provides an inconstant reference point. Preliminary studies indicated that total protein per unit wet weight also changed with development and so binding site density was normalized by tissue weight rather than homogenate protein. The relationship between tissue weight and protein concentration was established by a calibration curve.

Ventricles were dissected, weighed and homogenized in ice-cold HEPES solution (HEPES 20 mM, sucrose 250 mM, titrated to pH 7.8 with 1 M Tris base) using a Brinkmann Polytron at half maximal speed for 2 x 10 sec bursts. The whole homogenate was filtered through 4 layers of cotton gauze to remove large tissue fragments, and either used immediately or kept at -70°C prior to equilibrium binding assay. An aliquot of homogenate was taken to determine protein concentration by the method of Lowry et al. (1951) with a range of bovine serum albumin standards.

We also conducted equilibrium binding to purified myocytes in order to ensure that the contribution of beta-adrenoceptors in blood vessels to the specific $[^3H]$DHA binding in the ventricular homogenates was minimal. Myocytes were prepared from young adult hearts by perfusion with collagenase solution as described in detail by Hunter (1986).

6.2.2.2. Equilibrium binding

$[^3H]$DHA (specific activity: 42 Ci/mmol) was used as the specific ligand for the beta-adrenoceptors. Homogenate protein concentration curves and the rates of association and dissociation of $[^3H]$DHA from receptors was established to define the optimal conditions for the equilibrium binding assays (unpublished data). Total and non-specific $[^3H]$DHA binding was determined in the absence or presence respectively, of a large excess of propranolol (1 uM). Binding to ventricular homogenates (containing about 200 ug protein), or myocytes (35-40 x 10^3 cells; 250-300 ug protein), was initiated by adding aliquots of muscle homogenates to tubes containing a range of $[^3H]$DHA concentrations (0.3-30 nM) in a final volume of 200 ul, 50 mM Tris·HCl solution (pH 7.4). After incubation at 20°C for 30 min, aliquots of 10 ul from each tube were removed for determination of the total amount of radioactivity and subsequent calculation of the free [^{3}H]DHA concentration. The remainder was filtered through a Whatman GF/C glass filter (2.4 cm disc) and washed three times with 3 ml volumes of ice-cold Tris·HCl solution. The filters and 10 ul aliquots of incubated suspension were placed in plastic scintillation vials containing 10 ml Aquasol$^{(R)}$ and the radioactivity was measured using a Beckman Model LS 7500 liquid scintillation counter with quench correction. Presoaking the filters with 0.5% polyethyleneimine and 0.1 mM propranolol reduced the non-specific binding and provided specific binding with high resolution (Bruns et al., 1983; Bambrick et al., 1988; Chapter II).

6.2.2.3. Data analysis and statistics

The specific binding was determined by subtracting non-specific from total binding and was well fitted, using least mean square criteria, with a hyperbolic curve of the form

$$B = \frac{B_{max} \cdot [F]}{[F] + K_d}$$

where B_{max} is the maximum number of specific binding sites, K_d is the dissociation constant, and B is the amount of ligand bound specifically at [F], the concentration of free ligand, as shown in the examples in Figure 6.1A and 6.1B. Values of B_{max} and K_d were also calculated by replotting the data as a Scatchard plot (Figure 6.1C), where the x intercept is the B_{max} and the slope of the fitted regression line is $-1/K_d$. The B_{max} and K_d values obtained from hyperbolic curves were always in good agreement with estimates of the same parameters obtained from the Scatchard plots. The data were only acceptable where the regression lines were significant at the 1% level of confidence.

The B_{max} values from individual experiments were averaged for 4-8 preparations in each group, and expressed as arithmetic mean ± S.E.M. (Table 6.1 and 2). The geometric mean of K_d was obtained for the same preparations (Table 6.1 and 2) (Hancock et al., 1988). The equilibrium binding data from all the experiments in each group were also pooled and the B_{max} and K_d values obtained from the hyperbolic curves and Scatchard plots (see Figure 6.4) were in good agreement with the mean values from individual experiments. Only the mean values from individual experiments are shown in the Tables (see results section).

(Figure 6.1 near here)

Multiple comparisons of either mean values of B_{max} or K_d (arithmetic or geometric, respectively) were again carried out using Duncan's multiple range test for unequal sample sizes. Student-t-test was used for comparison of 2 groups in Table 6.2. Differences were taken to be significant where $P < 0.05$.

6.2.3. Materials

All ingredients of Krebs-Henseleit solution were of A.C.S. quality and obtained from Fisher Scientific Co. (Canada). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) with the following exceptions: Aquasol and [^{3}H]DHA were obtained from New England Nuclear (Boston, MA), and propranolol was the kind gift of Ayerst Laboratories (Montreal, PQ).

6.3. RESULTS

6.3.1. Inotropic responses of papillary muscles

At each stage of development, isoproterenol increased the tension developed by papillary muscles in a dose dependent manner (Figure 6.2A) without altering the time course of contraction. The maximal drug induced response, T_{max}, measured relative to steady state twitch tension at 1 Hz, increased sharply over the first 3 weeks of postnatal life and later declined to young adult levels (Figure 6.2A and 6.3A) which were still significantly higher than T_{max} in the newborn animal.

(Figure 6.2 near here)

When the concentration-effect curves were redrawn with the response to isoproterenol expressed relative to the maximum drug-induced response, it was clear that the sensitivity of the tissues increased with age (Figure 6.2B). This is seen by the progressive leftward parallel shift of the curves for the papillary muscles from the three age groups. The mean concentrations of isoproterenol required for half-maximal response (EC_{50}) were significantly lower ($P<0.05$) for muscles from the 3 week-old rabbits (6.6 nM, n=10) and young adult rabbit (2.8 nM, n=9), compared with muscles from 3 day-old rabbits (17.0 nM, n=8). The geometric means are shown in Table 6.1.

(Table 6.1 near here)

6.3.2. [^{3}H]DHA equilibrium binding to ventricular homogenates

Figure 6.1 shows that [^{3}H]DHA binds with high affinity to a single population of binding sites in young adult animals. Binding to homogenates in neonatal animals was also consistent with one population of sites. The data obtained from homogenates of ventricles were similar to that from homogenates of isolated myocytes (Table 6.2 and Figure 2.7), which indicates that there was little contamination of homogenates with non-cardiac beta-adrenoceptors.

(Table 6.2 near here)

As shown in Figure 6.3, B_{max} increases during neonatal life concurrent with the increase in the inotropic response to isoproterenol and thereafter, declines to the young adult levels. Note that the neonatal increase in numbers of beta-adrenoceptors is less than the corresponding increase in the inotropic response to isoproterenol, which shows that increased numbers of receptors does not fully account for the increased inotropic responsiveness of the ventricles. Although B_{max} and T_{max} decline from the high levels attained by 3 weeks of life, there is a net increase in adrenoceptor responsiveness during development since the young adult values of B_{max} and T_{max} are significantly higher than that in the newborn animals.

The error associated with the estimation of B_{max} in each experiment (see methods) is not taken into account in the arithmetic mean (±S.E.M.) shown in Figure 6.3. To further evaluate the significance of the developmental changes in B_{max}, we have pooled the data from all experiments in each age group in Figure 6.4. The pooled data at each age were fitted well with a single hyperbolic curve (Figure 6.4A, B and C) or with a straight line in Scatchard plots (Figure 6.4D, E and F) as for the single experiments (compare Figure 6.1). Note the 3-fold larger scale in the axes of specific [^{3}H]DHA binding from neonatal (Figure 6.4A and D) to 3 week old rabbits (Figure 6.4B and E) and thus the 3-fold increase in B_{max} from the newborn animal to young adulthood. The average values of B_{max} obtained in these composite curves in the figure agree well with the arithmetic means of B_{max} obtained from individual experiments (Table 6.1) which are significantly different in each age.

The slope of the regression lines for the Scatchard plots are similar in the neonatal animals (Figure 6.4D and E) but increases significantly in the young adult (Figure 6.4F) showing that the affinity of the adrenoceptors for the beta-receptor antagonists increases in the young adult heart. The average values of K_d obtained from the pooled data (see Figure 6.4) agree well with the mean K_d values (±S.E.M.) from individual experiments (Table 6.1).

(Figure 6.3 and 6.4 near here)

6.4. DISCUSSION

The present study indicates that two phenomena occur during postnatal development of the adrenergic responsiveness in the rabbit heart. There is a biphasic change in beta-adrenoceptor numbers, which increase for some time after birth and later decline to young adult levels. At the same time, the sensitivity of papillary muscle to beta-adrenoceptor agonists increases.

The biphasic change in the receptor numbers is consistent with data obtained from radioligand binding studies in the mouse (Chen et al., 1979; Roeske and Wildenthal, 1981). Other binding studies (see introduction) have excluded the neonatal period, and compared only hearts from juvenile and adult animals. These data suggested a decline in receptor numbers that would correspond to the second or declining phase shown in this study. This biphasic regulation of receptor numbers is confirmed by the corresponding biphasic change that we observed in inotropic response to isoproterenol. The latter was seen in the detailed study of contractility by Park et al.

(1980), but not detected when the adrenoceptor mediated inotropic response was studied at only two ages (Rockson et al., 1981; Baker and Potter, 1980).

The high B_{max} values measured at 3 weeks in the present work demonstrate the biphasic nature of development but do not necessarily identify the peak levels of beta-adrenoceptors. The number of receptors may either be declining or still rising at that time. Additional age groups would need to be added to the protocol to obtain a complete profile of the changes in receptor number.

These changes in adrenergic responsiveness undoubtedly involve influences operating on gene expression within the cell. One such influence may be the final maturation of the sympathetic innervation of the heart which occurs over the first three weeks of postnatal life in the rabbit (Friedman et al., 1968) and is coupled with increasing levels of circulating catecholamines (Pappano 1977). Thus the early rise in receptor numbers may be a reflection of unrestrained receptor synthesis by the cell prior to the maturation of innervation, when receptors will have little contact with adrenaline or noradrenaline. Following innervation, noradrenaline is released in appreciable quantities and would initiate the well-documented process of receptor down-regulation (Scarpace and Abrass, 1982; Roskoski et al., 1985) until an equilibrium state is reached.

Changes in inotropic response during development cannot be accounted for solely in terms of receptor numbers because the relative increase in the maximum inotropic responses between tissues from the neonatal and 3 week old rabbits is greater than the corresponding change in numbers of ligand binding sites. T_{max} increased by a factor of 5-fold in comparison with a 3-fold increase in numbers of $[^3H]$DHA binding sites (see comparison in Figure 6.3). Simultaneous maturation of the transduction mechanism that links receptor occupation to the contractile response of the cell could account for the increased inotropic response since the activity of the regulatory Gs protein and the catalytic subunits of the adenylate cyclase system increases in the first week of life in the rat and dog heart (Bhalla et al., 1980; Clark et al., 1980; Vulliemoz et al., 1984). Thus, the disparity between the change in relative inotropic response and B_{max} in tissues from 1-3 days and 20-22 day-old rabbits and the progressive increase in beta-adrenoceptor sensitivity may be due to an increasing efficiency of the transduction process. During further development, the later decline in the inotropic response occurs concurrently with a decline in the numbers of $[^3H]$DHA binding sites (this study), the activity of the Gs proteins and the adenylate cyclase (Bhalla et al., 1980). From the direct comparison of the inotropic response and number of beta-adrenoceptors in Figure 6.3, it is evident that a decline in the activity of the coupling is relatively more important. The inotropic responsiveness declines by 62%

compared with a 44% decrease in the number of receptors from the 3 week old to the young adult. Nevertheless, the adult inotropic responsiveness and the corresponding numbers of receptors are significantly higher than in the newborn ventricles. Thus the developmental increase in the inotropic responsiveness is, at least in part, a function of increased synthesis and incorporation of beta-adrenoceptors into the membranes of the ventricles.

Another possible explanation for this finding, although extremely difficult to test, is that there exists a receptor reserve in this system, and both absolute numbers of the receptors and the critical fraction which must be active to generate the maximum response, are both subject to change in development. This explanation is not formally much different from the explanation of changes in the coupling efficiency.

The progressive increase in sensitivity of papillary muscles to isoproterenol during maturation dose not apparently correspond to changes in the affinity for [^{3}H]DHA, as shown in Table 6.1. If both ligands occupy a common binding site (Caron et al., 1978), then the increase in tissue sensitivity to isoproterenol with development may reflect changes occurring in the efficiency of the transduction process, although the sensitivity of adenylate cyclase for adrenaline does not change during postnatal development (Vulliemoz et al., 1984). This hypothesis merits further experimental investigation. The decrease in the K_d value in the young adult is too small to account for the postnatal increase in the sensitivity of the receptor. The reason for the decrease in the K_d value within one order of magnitude is not clear, but may be clarified by more extensive investigation of affinity of the receptor for its agonists (see Bhalla et al., 1980). It is possible that changes in the membrane fluidity (Shinitzky and Barenholz, 1978), associated with the increase in the phospholipid-to-cholesterol ratio with age (Kutchai et al., 1976; Nagatomo et al., 1980), may influence the affinity of beta-adrenoceptors.

In summary, the biphasic increase in the adrenergic responsiveness of the rabbit ventricles with postnatal age is correlated to the corresponding changes in the beta-adrenoceptor population in the same tissues. In addition, the early increase in the adrenoceptor mediated inotropic response would also result from the increase in the efficacy of receptor-response coupling, and the second decline of the response may be a result of the receptor down-regulation following sympathetic innervation. The increase in sensitivity to beta-adrenoceptor agonists may serve to maintain a cardiac response to adrenoceptor agonists during maturation, at a time when there is significant down-regulation of the receptors concurrently with the increased coupling mechanism.

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Table 6.1. Comparison of beta-adrenoceptor mediated inotropy in papillary muscles and radioligand binding in ventricular homogenates from rabbits of different ages.

Postnatal age (days)	T_{max} (Isoproterenol) % increase $X\underline{+}S.E.M.$	B_{max} ($[^3H]$DHA) fmol/mg tissue $X\underline{+}S.E.M.$	EC_{50} (Isoproterenol) nM	K_d ($[^3H]$DHA) nM
1-3	36.7 ± 7.3	17.3 ± 2.3	17.4 (14.5 - 20.9)	9.12 (8.32 -10.0)
20-22	174.8 ± 32.4	56.6 ± 9.9	6.6 (4.36 -10.0)	6.34 (5.92 - 6.76)
80-100	67.9 ± 11.6	31.4 ± 4.5	2.8 (2.24 - 3.55)	4.07 (3.75 - 4.43)

Values B_{max} and T_{max} are expressed as the arithmetic mean ± S.E.M. from 6-13 experiments. T_{max} is the maximal increase in tension expressed as a % of the control tension generated in the absence of isoproterenol. Geometric means of EC_{50} and K_d are shown with the 5 and 95% confidence intervals given in parentheses. Differences in T_{max}, B_{max}, EC_{50} and K_d at the 3 different age groups are all significant ($p < 0.05$).

Table 6.2. Comparison of B_{max} (the arithmetic mean ± S.E.M) and K_d (the geometric mean with 5 and 95% confidence intervals) values for [^{3}H]DHA from adult ventricular homogenates and isolated myocytes.

Preparations	B_{max} (fmol/mg tissue)	K_d (nM)
Homogenates	31.4 ± 4.5	4.07 (3.75 - 4.43)
Myocytes	34.6 ± 4.4	3.98 (3.55 - 4.47)

The results were obtained from 4 - 8 experiments. B_{max} and K_d were not different in homogenate and myocyte preparations ($p > 0.05$).

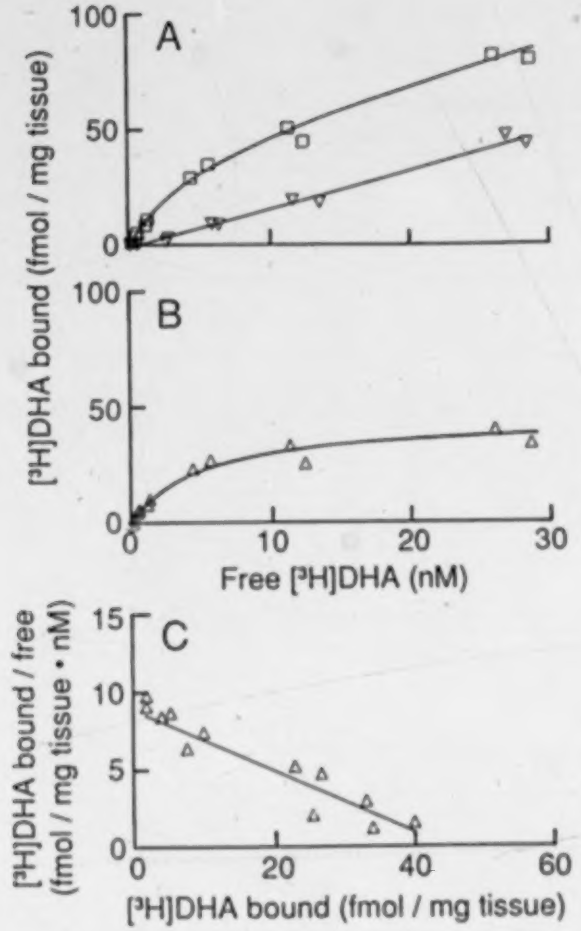

Figure 6.1. Equilibrium binding of [^{3}H]DHA to adult ventricular homogenates. A), Total (□) and non-specific bound (▽) of [^{3}H]DHA to the homogenates in the absence and presence of a large excess of propranolol (1 uM). The curve fitted to the total binding data is the sum of the line fitted to the non-specific data and the hyperbola fitted to the specific binding data in B using least-squares criteria (Hartley, 1961). B), the specific [^{3}H]DHA bound (△) was obtained by

subtracting the non-specific component from the total [^{3}H]DHA bound in A. The points were fitted, using least-squares criteria, with a hyperbolic curve of the form:

$$y = \frac{P_1 \cdot x}{P_2 + x},$$

where $P_1 = B_{max}$ and $P_2 = K_d$. C), Scatchard plot obtained by replotting the specific binding data. The intercept on the X axis is the B_{max} and the slope of the line is $-1/K_d$. In this particular experiment, B_{max} is 44.6 fmol/mg tissue·nM, K_d is 5nM and the slope ($\pm$ S.E.) of the regression line is 0.199 $\pm$ 0.02.

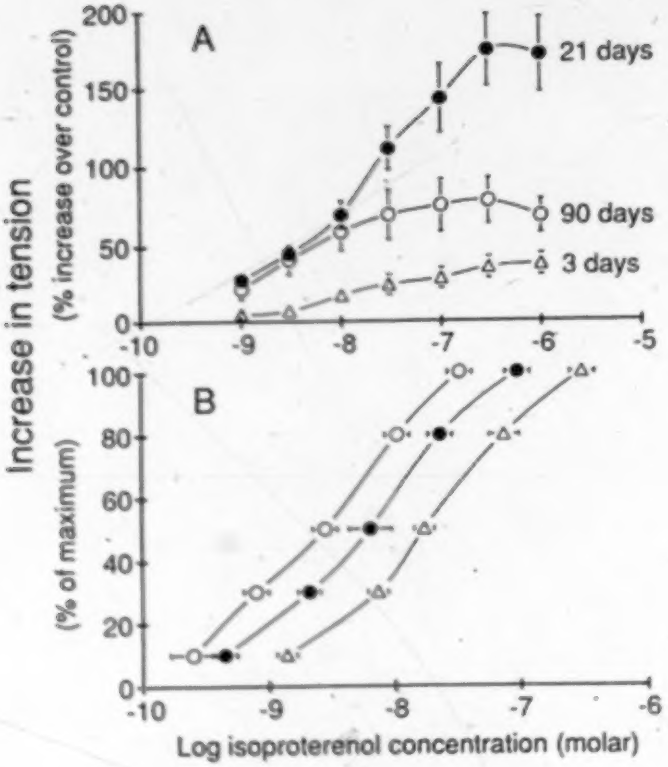

Figure 6.2. Mean concentration-effect curves of the positive inotropic response of papillary muscles to isoproterenol during postnatal development in the rabbit. A), the percentage increase in twitch tension with increasing isoproterenol concentration. The maximum responses increase dramatically from 1 - 3 days (△) to 20 - 22 days (●) old, and then decline to an intermediate adult level (○). B), the same data replotted as mean dose for given response. The parallel shift to the left of the curves with age indicates the sensitivity of the muscles to isoproterenol increases during postnatal development. Each point is the mean ± S.E.M. of 8 - 13 experiments.

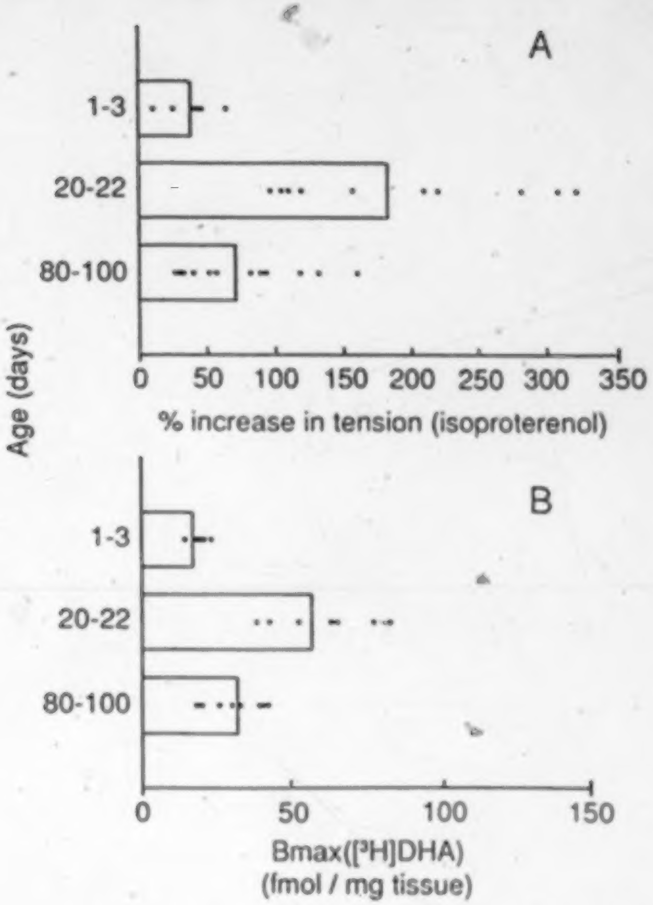

Figure 6.3. Comparisons of A), the maximum responses (% increase in tension) of papillary muscles to isoproterenol and B), the numbers of [^{3}H]DHA binding site (fmol/mg tissue) (B_{max}) in 1 - 3 days, 20 - 22 days and young adult of rabbit hearts. The bars represent the mean values and points, the actual values from individual experiments. The T_{max} increases from the newborn to 3 week old hearts by about 5-fold that contrasts to the 3-fold increase in the B_{max}. The increases in T_{max} and B_{max} from the newborn to adult hearts are identical. Duncan's multiple range test was used for unequal sample sizes comparison. Differences from other values in the same figure are significant ($P<0.05$).

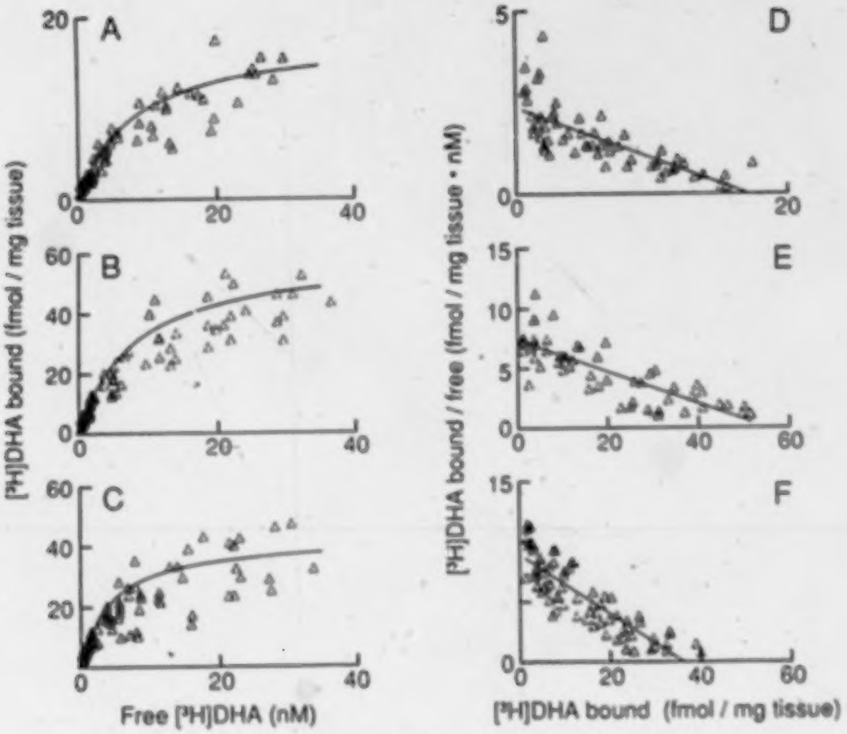

Figure 6.4. Specific [^{3}H]DHA binding to ventricular homogenates from different age groups. Data from individual experiments have been pooled in each group and fitted, using least-squares criteria either with hyperbolic curves (A, B and C) or with linear regression lines in Scatchard plots (D, E and F) as described in methods. B$_{max}$ and K$_d$ obtained from these composite curves agree well with average values obtained from individual experiments but provide a more realistic measures of variability. B$_{max}$ was 17.8 fmol/mg tissue in the newborn groups (A and D), 54.7 fmol/mg tissue in 3 weeks group (B and E) and 36.3 fmol/mg tissue in the adult (C and F), the slope of the regression lines in Scatchard plots were -0.11 ± 0.015, -0.12 ± 0.013 and -0.24 ± 0.016 in D, E and F, respectively.

CHAPTER VII. AUTONOMIC REGULATION OF RATE AND CONTRACTILITY OF ATRIAL MUSCLES DURING POSTNATAL DEVELOPMENT

7.1. INTRODUCTION

At the time of birth, the small cardiac reserve can be accounted for, in part, by relatively sparse internal Ca stores (Chapter V). We have found that these stores may be loaded to some extent by increase in frequency but the heart rate is already very high in the newborn (Gootman, 1983). Thus any modulation will depend on ability of autonomic nervous system to further increase the rate or contractile force. However, increased rate may be limited because of the strong tendency for arrythmia (Gootman et al., 1977; Gootman, 1983). The question arises as to whether the immature heart is modulated by chronotropic or inotropic responsiveness is predominant controlling the heart and what the relative balance of sympathetic and parasympathetic nervous systems are.

In contrast to the mature, the sympathetic nerve terminal in immature heart muscles of the newborn were sparse at birth and develops postnatally in rabbit (Friedman et al., 1968), lamb (Lebowitz et al., 1972), mouse (Mackenzie and Standen, 1976), rat (De Champlain et al., 1970) and dog (Kralois and Millar, 1978). In addition, noradrenaline concentration was low at birth and increased progressively during development (Iversson et al., 1967). Uptake of labelled noradrenaline was correspondingly low, and increased to adult levels over the first 4 to 5 weeks in the rat (Iversson et al., 1967; Atwood and Kirshner, 1976). Moreover, the young hearts had less capacity to store the catecholamines in comparison to the adult (Geis et al., 1975).

The vagal innervation of the heart was well established at the time of birth (Pappano, 1976). Nevertheless, the lesser acetylcholinesterase activity (Sinha et al., 1976, 1979) and small negative chronotropic effects of vagal stimulation (Mills, 1978) in neonatal hearts indicated that there is significant postnatal development.

The sarcolemmal Ca channel and beta-adrenoceptor are incorporated in ventricular muscles postnatally, both of which increase the cardiac reserve. It is possible that the increased capacity of ventricular contractile force occurs concurrently with a development of autonomic modulation of heart rate which together substantially increase cardiac reserve. This study shows that there is a dramatic fall in heart rate postnatally which occurs simultaneously with increased effectiveness of adrenergic modulation of atrial rate and force of beating.

7.2. METHODS

Right and left atria were dissected off from the heart of the 3 age groups of rabbits: newborn (1-3 day-old), juvenile (20-22 day-old) and adult (2-4 month-old). Each atrium was attached to a platinum wire hook and mounted in 4 ml tissue baths containing Krebs-Henseleite solution (pH 7.4) gassed with 95% O_2 and 5% CO_2, at 37°C. The atrial muscles were then connected to an Grass isometric force transducer by means of a length of suture thread.

The preload tension on the right atrium was adjusted to maintain the constant beating. The left atrium was stretched to the peak of its length-tension curve and then was relieved until 70% of peak tension reached. The transducers were connected to a pen recorder. The right atria beat spontaneously, the left atria were driven at 1 Hz of stimulation frequency with just suprathreshold pulses from a Devices isolated stimulator applied to platinum plate electrodes on either side of the tissue.

Preparations were allowed to stabilize for 1 h, after which the Krebs-Henseleit solution was changed and the tension readjusted. The spontaneously beating right atrial preparations were used to assess the chronotropic effects of both adrenoceptor and cholinergic receptor agonists, isoproterenol and methacholine, respectively, and the isometrically contracting left atria to assess the inotropic effects. Preparations were exposed to each drug concentration for 2 min to attain the maximal responses. Cumulative concentration-effect curves were obtained for each drug. After the maximum drug-induced responses were obtained, the preparation was washed until the rate or tension returned to its baseline value.

Results are expressed either as geometric mean (±S.E.M.) for EC_{50} and IC_{50} or arithmetic mean (±S.E.M.) for responses at each given drug concentration. Duncan's multiple range test was used to compare groups and difference was taken to be significant where $p<0.05$.

7.3. RESULTS

7.3.1. Beta-adrenoceptor mediated responses

Spontaneous beating rate of right atria decreased progressively during postnatal development (Table 7.1), and the rate of beating became more regular.

The rate of beating of the neonatal atria was very sensitive to isoproterenol. Threshold increases were recorded in response to 10^{-13} M isoproterenol (diluted in Krebs-Henseleit solution), which were not due to a volume change as the same volume of Krebs-Henseleit solution was ineffective in altering the atrial rate. The increase in the chronotropic effectiveness of isoproterenol with age is consistent with our findings in the ventricles that the density and receptor-effect coupling of beta-adrenoceptors increases with postnatal age (Feng et al., 1987, 1989; Chapter VI). The sensitivity to isoproterenol declined with increase in the maximal response which in the more mature atria Figure 7.1A). The maximal increase in responsiveness occurs during the first three weeks of postnatal life to reach adult levels (Figure 7.1A), even though heart rate continues to decline (Table 7.1).

(Table 7.1 and Figure 7.1 near here)

Similarly, when the same data are replotted as the geometric mean of the drug concentration against the percentage of maximal response, 10 fold parallel shift of the curve to the right from the newborn to the older groups shows that the change in sensitivity occurs during the first month of life (Figure 7.1B). Similarly, comparison of EC_{50} values in Table 7.2 shows the significant decline in sensitivity between newborn and the maturing muscles ($P < 0.05$).

(Table 7.2 near here)

The positive inotropic effects of isoproterenol (Figure 7.2) showed a different developmental pattern to the chronotropic effects. The inotropic effects of increasing the twitch tension of the paced left atria (1Hz) by a factor of 3 in the juvenile and then showed a small decline to the adult level (Figure 7.2A). The difference between the maximal increase in tension (T_{max}) in the juvenile to that in the adult was not statistically significant (Table 7.1).

(Figure 7.2 near here)

When the inotropic effects of isoproterenol were expressed relative to the maximum drug-induced response (as shown in Figure 7.2B), the concentration required for any response was not statistically different between each age groups showing that the beta-adrenoceptor sensitivity of left atria remains the same during postnatal development. The comparable EC_{50} values from each age group were shown in Table 7.2.

Thus, there is a significant developmental increase in the chronotropic and inotropic responsiveness of atrial muscles during the first month after birth. In the right atria there is an accompanying dramatic reduction in the sensitivity of the chronotropic response to isoproterenol in contrast to the inotropic sensitivity of the atrial muscles.

7.3.2. ACh-muscarinic receptor mediated responses

Unlike the beta-adrenoceptor mediated positive chronotropic effects, the negative chronotropic responsiveness of the atria was fully mature at birth. This is shown by the overlapping concentration-effect curves for methacholine in the right atria (Figure 7.3). IC_{50} values are tabulated in Table 7.2.

(Figure 7.3 near here)

In contrast, the negative inotropic effects of methacholine on left atrial muscles increased with age showing muscarinic modulation was not fully mature until 3 months of life (Figure 7.4).

(Figure 7.4 near here)

7.4. DISCUSSION

The substantial positive chronotropic and inotropic responses to isoproterenol in the atria in the newborn rabbit shows that the beta-adrenoceptor and underlying receptor-effector mechanism are already well developed at the time of birth. These findings are consistent with previous observations that chronotropic effects of catecholamines could be detected before birth in mice (Wildenthal, 1973) and chick (Loffelholtz and Pappano, 1974; Pappano 1976; for review see Sperelakis and Pappano, 1983). The finding of positive inotropic responses in atria is consistent with similar responsiveness in the ventricular muscles and detection of dihydroalprenolol binding sites in immature muscles (Chapter VI; Feng et al., 1989). The 3-fold increase in inotropic response is the same as that found in ventricular muscles where this increase was directly accounted for by a corresponding increase in a DHA binding sites.

In the ventricles, a secondary down-regulation of numbers of beta-adrenoceptors after 3 weeks of age was correlated with development of adrenergic innervation (Chapter VI) and may also be occurring in the left atria to account for a small decline in inotropic responsiveness in the adult relative to these from 3 weeks old juvenile. In the atria, on the other hand, we did not find a secondary decline but the tendency for arrythmia is developed at high isoproterenol concentration in the more mature muscles prevented resolution of the maximum response.

Postnatal decline in sensitivity of the right atria to isoproterenol suggests that the sympathetic innervation may also regulate the distribution of the incorporated beta-adrenoceptors. The immature muscles were extremely sensitive to isoproterenol even though the inotropic response was small. This could, by analogy with the high extrajunctional sensitivity of immature skeletal muscle to ACh, be due to a wide distribution of beta-adrenoceptors in the immature atria (Randall, 1979). Sensitivity declines as receptors are confined by the sympathetic innervation (Friedman, 1972). It cannot be explained by the immaturity of the presynaptic mechanisms for uptake of catecholamines (Iversson et al., 1967; Atwood and Kirshner, 1976; Standen, 1978) since isoproterenol is not affected (Hertting, 1964). However, it could be partly due to a postnatal increase in the activity of MAO, a major enzyme for catecholamine degradation (reviewed by Gripois, 1975).

The postnatal incorporation of beta-adrenoceptors contrasts with the apparent maturity of muscarinic-ACh-receptors into the heart after birth as indicated by constant chronotropic responses to methacholine in the right atria in immature and mature rabbit hearts and earlier findings of equivalent negative chronotropic effects of ACh in neonatal and adult hearts of canine (Danilo et al., 1973) and mouse (Wildenthal, 1973).

Numbers of [^{3}H]QNB (quinuclinyl benzilate, a specific muscarinic antagonist) binding sites to the muscarinic receptors are detected very early in the development of the chick heart (Sperelakis and Pappano, 1983) before precedes the development of the parasympathetic innervation (Gootman, 1983). This accounts for the chronotropic effective of ACh reported in fetal human and mouse atria (Schifferli and Caldeyro-Barcia, 1973; Wildenthal, 1973). Thereafter, the numbers of QNB binding sites decrease to reach adult levels (Nedoma et al., 1986).

Nevertheless, the negative inotropic responsiveness to methacholine shows a small but significant postnatal increase (Figure 7.4) despite the decline in muscarinic-receptor number (Nedoma et al., 1986). A possible explanation is that the maturation of receptor-effector coupling via G_i protein and an inhibition of adenylate cyclase continues during postnatal development. This is consistent with our findings that the maturation of adrenoceptor-effector coupling occurs postnatally even though numbers of receptors decrease (Chapter VI). The observations that the left atria undergo some postnatal maturation (inotropism) but not the SA node in the right atria (chronotropism), indicate different muscarinic subtypes and/or the receptor transduction system (Nedoma et al., 1986).

Thus, it is apparent that development of sympathetic and parasympathetic regulation of atrial function are not concurrent. Immature atria are extremely sensitive to adrenalin agonist, possibly due to a wide distribution of receptors but their rapid firing rate is little modulated by the innervation because of the low density of receptors and immature innervation (De Champlain et al., 1970; Owman et al., 1971). In contrast, parasympathetic modulation is mature at birth and is effective in reducing the high rate of beating in the immature neonatal heart.

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Table 7.1. Comparison of chronotropic effects of isoproterenol and methacholine in right atria from postnatal developing rabbits.

	Atrial rate (beat/min)		
Postnatal age	Control	Isoproterenol (10^{-8} M)	Methacholine (10^{-5} M)
1-3 day-old	248 ± 7	305 ± 3	96 ± 18
3 week-old	213 ± 8*	298 ± 17	99 ± 11
3 month-old	184 ± 10*	257 ± 21*	59 ± 20

The data were expressed as arithmetic mean (± S.E.M.), from 6 - 13 preparations.

* significant different to the neonatal value at the same column ($p < 0.5$).

Table 7.2. Comparison of inotropic effects of isoproterenol and methacholine in left atria from postnatal developing rabbits.

Postnatal age	EC_{50} (isoproterenol)		IC_{50} (methacholine)
	Right atria	Left atria	Right atria
1-3 day-old	-11.45±0.17	-8.04±0.22	-5.68±0.23
3 week-old	-9.42±0.34*	-8.55±0.33	-5.37±0.13
3 month-old	-9.78±0.25*	-8.28±0.14	-5.67±0.26

Logarithmic EC_{50} and IC_{50} values were expressed as arithmetic mean (± S.E.M.) from 6 - 9 preparations.

* significant different to the neonatal value at the same column ($p < 0.5$).

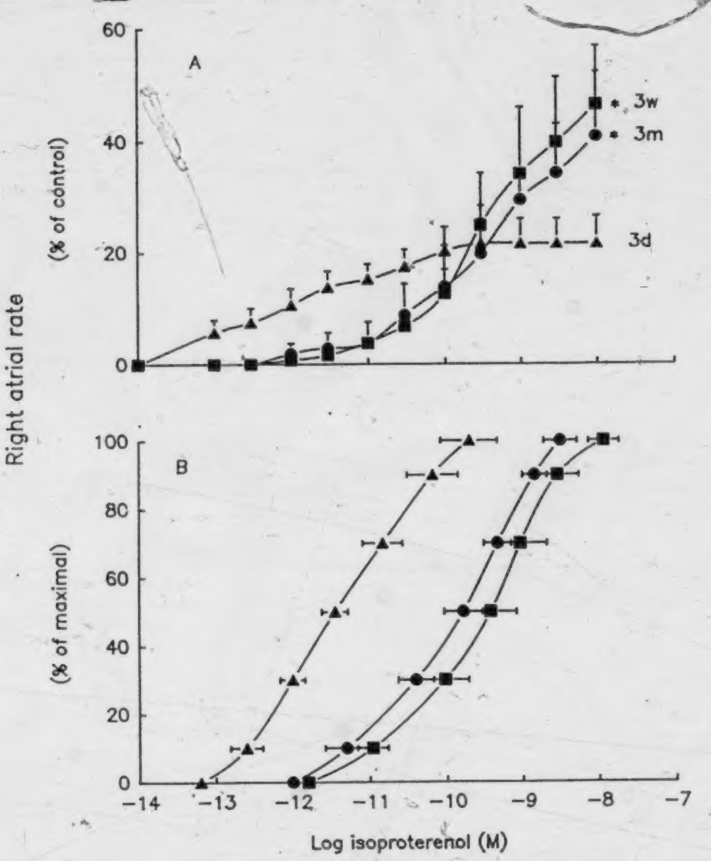

Figure 7.1. Mean concentration-effect curves of the positive chronotropic effects of isoproterenol on right atrial muscles from different age groups of rabbits. A), The percentage increase in contractile rate over the control rate recorded in the absence of isoproterenol was plotted as function of isoproterenol concentration. B), The same data were replotted as mean concentration for given response. The concentration-effect curve in the neonatal atria shifted parallelly to the left which indicated the sensitivity of the neonatal muscles to isoproterenol was greater than that of the older groups. Each point is the mean ± S.E.M. of 6 - 8 experiments.

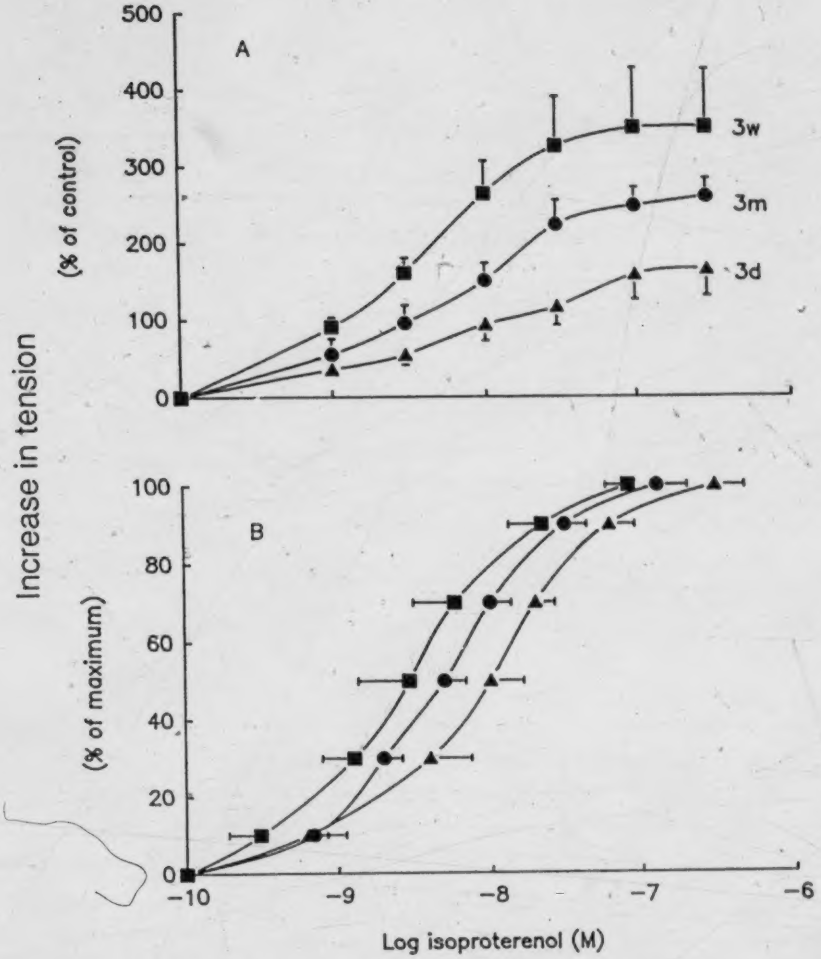

Figure 7.2. Mean concentration-effect curves of the positive inotropic effects of isoproterenol on left atrial muscles from different age groups of rabbits. A), The percentage increase in contractile tension over the control tension recorded in the absence of isoproterenol was plotted as function of isoproterenol concentration. B), The same data were replotted as mean concentration for given response. There was no different in each age group. Each point is the mean ± S.E.M. of 6 - 9 experiments.

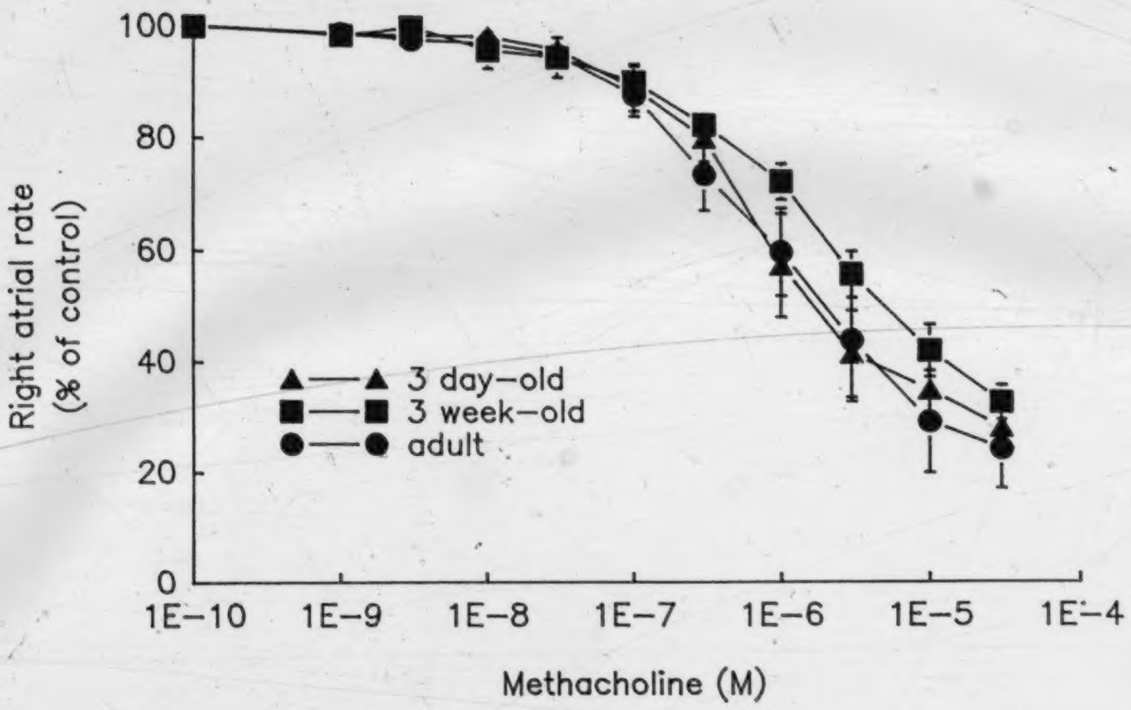

Figure 7.3. Mean concentration-effect curves of the negative chronotropic effects of methacholine on right atrial muscles from different age groups. The data were plotted as the percentage decrease in atrial rate of the control rate which was obtained in the absence of methacholine, and no difference was found in the developing muscles. (n = 5 - 7).

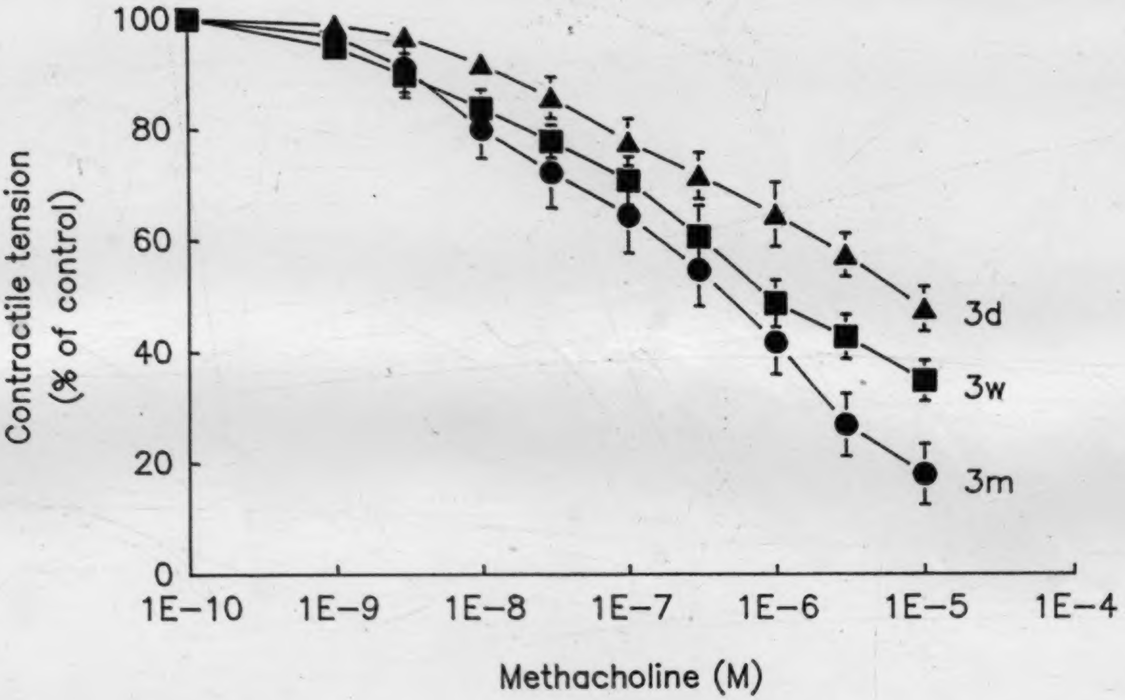

Figure 7.4. Mean concentration-effect curves of the negative inotropic effects of methacholine on left atrial muscles from different age groups. The data were plotted as the percentage decrease in contractile tension of the control tension which was obtained in the absence of methacholine. The methacholine induced inhibition on the contractile tension increased progressively with age. (n = 5 - 7).

CHAPTER VIII. DISCUSSION

Myocardial contractile function in the rabbits undergoes postnatal development. The present studies demonstrated that the capability of the cardiac muscles to develop maximum contractile force and modulation by tissue length, stimulation frequency and various positive inotropic agents including Bay K8644 (a sarcolemmal Ca channel agonist) and isoproterenol (Beta-adrenoceptor agonist), was less in the neonatal rabbit and increased as the animal matures. These data are consistent to the findings by other investigators in lambs (Romero and Friedman, 1979; Rudolph and Heymann, 1974; Teitel et al., 1985) and in rabbits (Romero et al., 1972; Friedman, 1972) which showed an age-dependent increase in myocardial contractile reserve during postnatal development.

Morphological studies have indicated that the limited capability of the immature heart to develop contractile force may be due to its structural features which include less contractile and more non-contractile (water content, nuclear materials and connective tissues) elements as compared to that of the adult (Yao, 1983; Gilbert, 1980). However, functional studies demonstrated that the resting cardiac output (Klopfenstein and Rudolph, 1978; Berman and Musselman, 1979; Gilbert, 1980) or the resting myocardial contractility (Teitel et al., 1985) was high in the newborn heart and reduced during postnatal development. Therefore, postnatal increase in either amounts of contractile proteins or the intrinsic activity of myofibrillar ATPase in heart muscles (Nayler and Fassold, 1977; Maylie, 1982) are not sufficient to explain the enhanced contractile reserve, since that the newborn may be functioning at its maximal contractile capacity after birth when physiological demand is high, but it is unable to substantially increase its myocardial performance. Despite these conflicting observations, the present studies indicate that limitation of contractile reverse in the neonatal heart is due, at least in part, to postnatal maturation of the processes which control Ca compartmentalization and E-C coupling.

It is most likely that contribution of intracellular stores to contractile Ca in the neonate heart is insufficient since the structure and function of the SR are relatively immature at the time of birth (Chapter IV and V). The contraction in the immature heart depends mainly on external Ca which enters the cell via the sarcolemmal Ca channels during the action potentials. However, the number of the Ca channels in the membrane of maturing hearts is small in the neonate (Chapter III). Thus the elevation of the Ca concentration in the cells via Ca channels during E-C coupling is limited in the newborn heart, this, in turn, limits contractile reserve since the force of contraction is proportional to the concentration of free Ca in the interior of the cell (McCall, 1987). The progressive development of the SR and its ability to exchange Ca between intracellular and extracellular Ca compartments will increase the reserve of the maturing heart.

The studies of sympathetic modulation of cardiac contraction indicate that beta-adrenoceptor mediated positive inotropic effects increase concurrently with the receptor population during postnatal development (Chapter VI and VII). Although these differences are correlated to the age-dependent sympathetic innervation (Kralois and Millar, 1978) and receptor-effector transduction system (Vulliemoz et al., 1984), the smaller inotropic effects of beta-adrenoceptors in the neonatal hearts is due in part to the immaturity of their Ca transport mechanisms.

Development of E-C coupling in atrial muscles has progressed further than papillary muscles at the time of birth. The intracellular Ca stores in atrial muscles are more mature from the point of view of the function of ryanodine-sensitive Ca release sites (Chapter V). The findings that maximal inotropic responses of the atrial muscles to Bay K8644 and isoproterenol are always greater than that of the papillary muscles in each age group (Chapter III, V) provide the further evidence that the myocardial contractile reserve is related to the extent of intracellular Ca stores. Increases in maximal positive inotropic effects of the drug in the atria during postnatal development, however, may be account for by an increase in the capacity of the SR to load Ca.

Since the contractile reserve is limited at birth, the high cardiac output required to meet the increased demand of the growing animal after birth must depend more on heart rate than stroke volume. With time, stroke volume becomes more capable and contributes more to maintain constant cardiac output. The autonomic modulation of cardiac output, at the same time, is more significant to meet physiological and even pathological demands.

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